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Cellular and Molecular Actions of Juvenile Hormone. II. Roles of Juvenile Hormone in Adult Insects

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1 Introduction

The juvenile hormone, first discovered by Wigglesworth (1934, 1936), plays crucial roles in the development and reproduction of insects. During larval and pupal development, it governs metamorphosis, modifying the actions of 20-hydroxyecdysone (20HE) so as to inhibit the development of adult characters. At a critical stage early in the final moult, JH disappears,

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We dedicate this review to the late Sir Vincent Wigglesworth, FRS, who first drew attention to the corpus allatum and juvenile hormone, and whose research in insect physiology and endocrinology over more than six decades both illuminated the subject and inspired generations of researchers.

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permitting metamorphosis to take place. In the adult, however, the corpus allatum (CA) which produces the hormone once again becomes active and JH governs a wide variety of functions related to reproductive maturation and function in a range of tissues in both the female and the male. While several other hormones are involved in the control of reproduction, it is JH that plays the principal role of coordinating the activities of the various tissues so that the gametes are produced, and the behaviours related to mating and oviposition occur at the appropriate times. This involves responses to JH in the fat body, the nervous system and even in some cases the muscles, as well as the gonads and accessory reproductive glands. Various environmental factors such as food, mating, temperature and photoperiod exert their effects on reproduction through JH, and the CA is subject to a network of neural and humoral controls which may be complex (Tobe, 1980).

The structure of this remarkably pleiotropic hormone is unique among animal hormones. JH was first isolated from the abdomens of male cecropia moths as a crude oil (Williams, 1956), and the chemical structure of the active component emerged as an epoxidated ester of a homologue of farnesoic acid (Röller *et al.*, 1967). Several other forms of the hormone have since been identified, differing by the lengths of the side chains (JH I = C18, JH II = C17, JH III = C16, JH 0 = C19, each with a 10,11-epoxide link). More recently, a derivative of JH III with an additional 6,7-epoxide, designated JHB3 has been identified from higher Diptera (Richard *et al.*, 1989; Cusson *et al.*, 1991; Lefevere *et al.*, 1993), although its biological role has yet to be elucidated. There is evidence that methyl farnesoate may function as a JH in Crustacea (Borst *et al.*, 1987; Laufer *et al.*, 1993). The structures of these various JHs are shown by Riddiford (1994).

The consensus from many studies is that JH I and JH II are found primarily in the Lepidoptera, while most other insects use JH III (Schooley *et al.*, 1984). The identity of the JH molecule active in Hemiptera remains in doubt. *Oncopeltus fasciatus* appears not to contain any of the known JHs (Baker *et al.*, 1988). JH I has been identified by GC/MS from the haemolymph of the bean bug, *Riptortus clavatus* (Numata *et al.*, 1992), but the principal, biologically active product from incubation *in vitro* of the CA of this and other Hemiptera does not appear to correspond to JH I or any known JH (Kotaki, 1993, 1996).

In the search for 'Insect Growth Regulators' with potential value in pest management, numerous compounds have been synthesized that show JH-like activity in a variety of assays (Slama *et al.*, 1974; Staal, 1975; Sehnal, 1983; Retnakaran *et al.*, 1985). The structures of several highly active compounds are shown by Riddiford (1994). Some of them, such as methoprene, share with JH a modified terpenoid structure, although lacking features such as the epoxide group, which is essential for biological activity in the natural hormone. Others of these 'juvenoids' bear little resemblance to the JH molecule but include phenoxyphenoxy derivatives such as fenoxycarb and

pyriproxyfen. Since such compounds are widely used in research because of their stability and often greater biological activity than natural JHs, the question to what degree they truly function as JH agonists is crucial.

When presumed JH analogues are applied in low doses to allatectomized insects and are found to restore processes that were abolished by removal of the CA, and that can also be restored by JH itself, it is reasonable to conclude that they are acting at the same sites as JH. Many examples that fulfil these criteria will be cited in this review. For example, on this basis methoprene and pyriproxyfen are believed to act as JH agonists in precocene-treated adult locusts (Edwards *et al.*, 1993). It is important, however, to be aware that these active compounds, especially when used in high doses, can have other effects. In insects with intact endocrine systems, they may affect the production of JH in the CA (Tobe and Stay, 1979), and (before metamorphosis) that of ecdysone from the prothoracic glands (Steele and Davey, 1985; Watson *et al.*, 1987). A recent review on fenoxycarb (Grenier and Grenier, 1993) concludes that this compound has a high JH activity but also has other non-JH-specific targets, for example inhibiting JH esterase in insects and influencing nucleic acid metabolism in protozoa and human lymphocytes (which presumably have no JH receptors). In honey bees, wax secretion was inhibited by methoprene, even though neither allatectomy, implantation of active CA, nor application of JH III had any effect on this process (Muller and Hepburn, 1994). In addition, the extraordinary differences in activity of various JH analogues in bioassays on different insect species remain unexplained. The situation may be analogous to that of the non-steroidal ecdysteroid agonists, where the case is persuasive for dibenzoyl hydrazines acting at the ecdysone receptor, but they can also exhibit neurotoxicity by mechanisms unrelated to ecdysteroid-like action (Oberlander *et al.*, 1995).

A curious aspect of the search for ever more powerful JH analogues is the failure to discover effective JH antagonists that clearly act competitively at the sites of JH action (Staal, 1986). This eventuality may be understood only when the molecular mechanisms of primary JH action are known. However, the plant chromene, precocene, and certain derivatives of it are effective in blocking JH production in many insects through specific cytotoxic action on the CA (Bowers *et al.*, 1982). Although some non-specific effects of precocenes have been recorded, they are valuable and widely used in research as an alternative to surgical allatectomy.

While there is abundant research on the biological effects of JH in adult insects, there is a paucity of studies on the mechanisms underlying these actions, especially when compared with the intensive research on the molecular genetics of ecdysteroid action. A valuable background coverage of the roles of JH in insect reproduction is found in the chapter of Koeppe *et al.* (1985), and an overview of the roles of JH and other regulatory factors is provided by Raabe (1986). The cellular and molecular actions of JH have

been considered in a general context by Kumaran (1990) and recently reviewed in detail for premetamorphic insects by Riddiford (1994). Another recent review focuses on the conceptual and experimental approaches to JH action in the nucleus (Jones, 1995). In the present article, we shall review the specific actions of JH in adult insects, explore the evidence related to its cellular and molecular modes of action, and indicate the major problems that remain. Although it has been an implicit assumption in much of the research that there is a unique site of action for JH, that view is no longer tenable. We will show that there may be more than one site of action, even in a single cell.

2 Biology of juvenile hormone action in adult insects

In various insects, the degree to which the timing of reproductive development and activity is responsive to external signals varies greatly. In some, such as silk moths, gametogenesis is complete at the time of eclosion and the adults are ready for immediate mating and oviposition, with no need for hormonal regulation. In many insects, for example cockroaches, locusts and mosquitoes, the adults are programmed for early completion of oogenesis, but the timing is regulated by food intake and other signals which act through their influence on JH titres. In still others, reproduction can be delayed or repressed in response to environmental signals (notably photoperiod and temperature) that foretell the onset of an unfavourable season, bringing the insects into a state of reproductive diapause which may last for an extended period of time. The earlier work on insect diapause has been thoroughly reviewed by Denlinger (1985), who gives a list of species in which adult diapause is brought on by JH deficiency and can be interrupted by JH or a JH analogue. Among more recent reports, reproductive diapause has been shown to be induced by lack of JH and broken by administration of JH or an analogue in two species of plant bugs (Numata and Hidaka, 1984; Kotaki and Yagi, 1989), and a review has appeared on reproductive diapause in adult males (Pener, 1992). The role of JH in adult diapause has been especially thoroughly studied in the Colorado potato beetle, *Leptinotarsa decemlineata* (de Kort *et al.*, 1987; Koopmanschap *et al.*, 1989; de Kort, 1990). In adult diapause of different species, while the gonads and accessory glands undergo involution, the insect may become quiescent, or it may engage in non-reproductive activity such as migration (Pener, 1985). In both situations, a major coordinating role is played by JH, which mediates the signal to the various organs that are involved, including the gonads, accessory glands, fat body, muscles and ganglia that control behaviour patterns.

Another important phenomenon in which JH is clearly involved, although it is not yet clear just how, is phase polymorphism (polyphenism). This is

well exemplified in the desert locust (*Schistocerca gregaria*) and the migratory locust (*Locusta migratoria*), both of which exhibit a solitary phase under conditions of low population density, but are transformed over several generations to a gregarious phase when conditions become favourable for reproduction and dense populations build up. The two phases differ in morphology, colour pattern, physiology and behaviour: whereas solitary individuals are relatively sedentary and the adults mature more rapidly for reproduction, crowded locusts are restless, gregarious and prone to migrate in swarms. Since these swarms are responsible for devastation of agriculture, the causes of the phase transformation have been the subject of much research (Uvarov, 1996, 1977). Several of the characteristics of the solitary phase, such as green colouration and early reproductive maturity, can be brought on by JH or analogues applied to late hoppers or early adults, and it has been proposed that elevated JH is the determining factor that induces solitarization (Nijhout and Wheeler, 1982). Two recent papers interpret physiological and behavioural consequences of application of JH or analogues to gregarious *S. gregaria* as solitarization effects (Schneider *et al.*, 1995; Wiesel *et al.*, 1996). A thorough review of the evidence, however, has led Pener (1991) to conclude that altered JH production is only one aspect of the process of phase transformation, which is brought about by altered environmental conditions through complex mechanisms that are not yet understood. For example, the development of yellow colour in reproductively mature locusts is controlled by JH: it is prevented by allactectomy and restored by application of JH or analogues. Yellowing, however, is observed only in gregarious phase individuals, and is not induced in solitaries by any regimen of JH, whereas transfer to a crowd is followed by yellowing (Pener, 1991). It is of interest to note here that the integumentary yellowing presumably represents an effect of JH upon the epidermis, a tissue well known as a JH target in premetamorphic insects (Riddiford, 1994), but which has not been widely documented as a JH target tissue in adults.

In the following sections, we shall review the reported effects of JH, more or less directly related to reproduction, in several organs of the adult insect. We shall emphasize instances where specific cellular activities and gene products have been identified.

2.1 THE FAT BODY

The fat body plays many essential roles in insect physiology and development (Wigglesworth, 1972; Locke, 1980), and as a target tissue for several important developmental and homeostatic hormones, it is well suited for the study of mechanisms of hormone action (Keeley, 1978). The fat body consists of thin branching lobes, dispersed in a manner characteristic for each species,

most abundant in the abdomen but present also in the thorax and head. These comprise principally a single cell type (adipocytes or trophocytes), although in cockroaches and some other insects specialized mycetocytes and urate cells are also present. The cells are separated from the haemolymph only by a thin connective tissue sheath which invests each lobe, and there is ready exchange of metabolites and more selective exchange of proteins with the haemolymph. At metamorphosis, in the majority of insects much of the fat body survives with appropriate reconstruction to the adult stage, but in some Hymenoptera and the higher Diptera the larval fat body undergoes histolysis and a new adult fat body arises from dedicated histoblasts or from some surviving larval fat body cells.

The fat body has three principal roles: intermediary metabolism (including carbohydrates, lipids and nitrogenous compounds), storage of reserves (and sometimes wastes) and the production of haemolymph proteins. The cells undergo huge changes in content of fat droplets, glycogen and protein during feeding and starvation, moulting and metamorphosis. Especially during the last larval instar of endopterygote insects, reserves are accumulated to provide for the development of adult organs without further intake of food, while the fat body cells become polyploid and enlarge. Of particular interest from the point of view of endocrine regulation are the hexameric storage proteins that are produced in the fat body during the feeding phase of the last larval instar to provide amino acid reserves for the subsequent construction of adult tissues, and some storage proteins are also produced in adults (Section 2.1.4.1). After metamorphosis, among the major products of the fat body are the vitellogenins (Vg) and other yolk precursor proteins, the JH-regulated synthesis of which provides good systems for the analysis of JH action (Sections 2.1.1, 2.1.2, 2.1.3). Effects of JH have also been reported on some haemolymph proteins that do not contribute substantially to the egg (Section 2.1.4). Another abundant haemolymph protein coming from the fat body is the lipid transport protein, lipophorin (Shapiro *et al.*, 1988), the synthesis of which, at least in certain insects, appears to be unaffected by JH (Section 3.5.4.1).

The most intriguing aspect of this picture is the developmental alteration of fat body cell function (Locke, 1980). In the Exopterygota and the less specialized of the Endopterygota, the same fat body cells switch impressively between principally storage and predominantly protein-synthetic roles, in the selection of genes for active expression, and in their responsiveness to JH and other hormones. Cells that are identical in structure and most functions show sex-specificity in expression of certain genes, and this sex-limitation may change with development. For these reasons, the fat body of hemimetabolous insects has been nominated as a 'minimal model of metamorphosis' (Kunkel, 1981). With the recent flowering of molecular genetic methodology, the tools now exist for analysis of the molecular basis of these regulatory mechanisms.

2.1.1 *Vitellogenins*

The yolk precursor proteins named vitellogenins (Vgs) by Pan *et al.* (1969) have received much attention as subjects for study of regulation by JH in postmetamorphic insects (reviews: Hagedorn and Kunkel, 1979; Engelmann, 1979, 1983; Chen and Hillen, 1983; Postlethwait and Giorgi, 1985; Kunkel and Nordin, 1985; Koeppe *et al.*, 1985; Bownes, 1986; Kanost *et al.*, 1990; Wyatt, 1991). They are a superfamily of high molecular weight glycolipoproteins, encoded by mRNAs of 5.5–6.5 kb and translated as polypeptides of 215–260 kDa, which are processed within the fat body by proteolytic cleavage and addition of carbohydrate, phosphate, sulfate and lipid (Bownes, 1986; Dhadialla and Raikhel, 1990). Several insect Vgs have been cloned and shown by DNA sequencing to be homologous with the Vgs of other invertebrates such as nematodes and with those of oviparous vertebrates such as amphibia and birds (Speith *et al.*, 1985; Blumenthal and Zucker-Aprison, 1987; Nardelli *et al.*, 1987; Wahli, 1988; Byrne *et al.*, 1989; Trewitt *et al.*, 1992; Chen *et al.*, 1994). Similarity of the proteins also suggests homology with Vgs of other invertebrates, such as ticks (Chinzei *et al.*, 1983; Rosell and Coons, 1991), millipedes (Prasath and Subramoniam, 1991), Crustacea (Chen and Chen, 1993) and sea urchins (Shyu *et al.*, 1986).

Insect Vgs are produced chiefly in the fat body and transported via the haemolymph to the ovary, although in a few species synthesis within the ovary has also been reported (*Coccinella*: Zhai *et al.*, 1985; *Thermobia*: Rousset and Bitsch, 1989; see also Section 2.2.1.2). In most insects, both the synthesis in the fat body and uptake into the ovarian follicle are dependent on stimulation by JH. Among other arthropods, the JH homologue, methyl farnesoate, seems to have roles in the regulation of crustacean reproduction which may include stimulation of Vg synthesis (Laufer *et al.*, 1993). In certain ticks some evidence has been reported for participation of JH or a related compound in regulating egg development, but recent experiments do not support this conclusion and indicate that other hormones, possibly including ecdysone, are involved (Oliver and Dotson, 1993; Lunke and Kaufman, 1993; Chinzei and Taylor, 1994).

Among the insects, regulation of Vg synthesis by JH is commonly, but not universally, observed, and the endocrinology is adapted to the biological needs of different insects (Section 2.1.1.10). In some species, there is evidence that neurosecretory products from the brain can work synergistically with JH in the regulation of Vg synthesis (Section 3.5.4.6).

The earlier reports that demonstrate participation of the CA or their endocrine product in regulation of vitellogenesis (including both synthesis and uptake of yolk protein) have been listed in previous reviews (Wyatt, 1972; Engelmann, 1979, 1983). Here, we shall review some of the key discoveries together with more recent findings, for insects belonging to different orders.

2.1.1.1 *Thysanura.* In this primitive apterous order, vitellogenesis in *Thermobia* (firebrat) depends on the CA and is inhibited by treatment with the anti-allatal compound, precocene (Bitsch and Bitsch, 1984). Since moulting continues in the adult stage, oogenesis is coordinated with the ecdysone-regulated moulting cycle (Bitsch *et al.*, 1986). The Vg has been characterized and its haemolymph titre during a reproductive cycle shown to exhibit a peak which is elevated after insemination (Rousset *et al.*, 1987, 1988).

2.1.1.2 *Dictyoptera.* The cockroaches were among the first insects in which an effect of the CA on production of Vg was demonstrated, and several species have been used intensively for research in this area. In the oviparous cockroach, *Periplaneta americana*, the ovarian gonotrophic cycles, including both the synthesis and uptake of Vgs, are regulated by the CA (see reviews cited above; also, Chen *et al.*, 1962). After inhibition or removal of the CA, ootheca production could be stimulated by administration of the JH analogues, hydroprene or fenoxycarb (Edwards *et al.*, 1985; Weaver and Edwards, 1990). In *Blattella germanica*, the growth of terminal oocytes has been shown to respond quantitatively to different doses of JH I and analogues (Kunkel, 1973), and several aspects of the cellular physiology of the Vg have been studied (Kunkel and Nordin, 1985). A recent study on *B. germanica*, using ELISA to assay Vg in fat body, haemolymph and ovaries, concluded that Vg production is not quantitatively correlated with JH titre, and indicated that other factors are crucial in the initiation and termination of Vg synthesis (Martin *et al.*, 1995a; see Section 3.5.4.6). In this species, as in *Locusta* (Chinzei and Wyatt, 1985), ovariectomy is followed by a large accumulation of Vg, and from this observation, Martin *et al.* (1995b) suggest that an ovarian signal acts on the fat body to terminate vitellogenesis in the face of rising titres of JH.

In females of the ovoviviparous cockroach, *Nauphoeta cinerea*, Vg production could be induced by injection of methyl farnesoate (farnesyl-methylester) in penultimate or last instar larvae, or adults, with the strength of response successively increasing and requisite dose decreasing through these developmental stages (Lanzrein, 1974). Subsequently, JH I was shown to be about 100-fold more active than methyl farnesoate (Buhlmann, 1976), and JH II and JH III slightly more active than JH I (Lanzrein, 1979), for Vg induction in adult female *N. cinerea*. Further studies showed that a much greater dose of JH III is required to stimulate oocyte growth than Vg synthesis, and led to a model for interactions of JH, the CA and the ovary in the initiation of an oocyte maturation cycle (Buschor *et al.*, 1984).

In *Blaberus discoidalis* (ovoviviparous), two Vgs have been identified and shown to be produced in the fat body, and in nonvitellogenic ovulated female adults their synthesis was induced by administration of massive doses of JH III (Wojchowski and Kunkel, 1987). In decapitated female adult *B.*

discoidalis, prolonged treatment with methoprene or JH III induced the synthesis of Vg, assayed both as the protein and as 6.5 kb mRNA, while repressing production of two other proteins (Sections 2.1.4.1, 2.1.4.4; Keeley *et al.*, 1988; Bradfield *et al.*, 1990; Jamroz *et al.*, 1993; Fig. 9). This system has potential for molecular analysis of transcriptional regulation. With *Blaberus craniifer*, however, in which vitellogenic cycles are normally correlated with elevated JH, yolk deposition was reported in oocytes of a certain proportion of decapitated females after implantation of glass beads into the haemocoel, or after ablation of cerci, thus indicating the existence of regulatory mechanisms independent of the neuroendocrine organs of the head (Goudey-Perrière *et al.*, 1989; Brousse-Gaury *et al.*, 1990).

In the viviparous cockroach, *Diploptera punctata*, removal of the CA prevented the normal appearance of Vg in adult females (Mundall and Tobe, 1979). In adult males, Vg, which is not normally present, could be induced by implantation of CA, or by topical application of hydroprene (Mundall and Tobe, 1979; Mundall *et al.*, 1983). The level of haemolymph Vg produced after CA implantation was lower in males than in females. In the brood pouch of this species, allatectomy caused a delay in parturition and in the normal pre-parturition decline in milk production, and these effects could be reversed by application of hydroprene (TerWee and Stay, 1987).

In another viviparous species, *Leucophaea maderae*, fat body Vg synthesis was early shown to be strictly dependent on juvenile hormone or analogues (Engelmann and Penney, 1966; Chambers and Brookes, 1967). ED_{50}s recorded are 1 μg for JH I topically applied in olive oil (Engelmann, 1971), or about 25 μg for JH III topically in acetone (della-Cioppa and Engelmann, 1984b); methoprene is also effective (Don-Wheeler and Engelmann, 1991) but the ED_{50} is not recorded. This species has been used for studies on the biochemical processes involved, to be discussed in Section 3.5.

2.1.1.3 *Orthoptera.* In several species of grasshoppers and locusts (Acrididae), experiments in the 1960s and early 1970s involving extirpation and transplantation of CA indicated that JH is essential for vitellogenesis (see reviews cited above; also Gillott and Elliott, 1976; Elliott and Gillott, 1977). This has more recently been confirmed in *Melanoplus bivittatus*, and the fat body of this species has been proposed as a model for the study of JH action (Roberts and Jefferies, 1986). The regulation of Vg synthesis by JH in *Locusta migratoria* has been studied in detail as an approach to analysis of the molecular biology of JH action, and the findings on this system will be discussed later in this review (Section 3.5). Induction of Vg synthesis in *Locusta* by JH I required repeated doses (Chen *et al.*, 1979) and by JH III was achieved only with a high dose and simultaneous injection of a JH esterase inhibitor (Wyatt *et al.*, 1987). With the synthetic analogues, on the other hand, strong induction was regularly obtained after a single application, either injected in oil or applied topically to the neck in acetone, with the

following approximate ED_{50}s: 7*S*-methoprene, 30 μg; pyriproxyfen, 2 μg, fenoxycarb, 15 μg (Dhadialla and Wyatt, 1983; Edwards *et al.*, 1993; G. Edwards and G. Wyatt, unpublished). A recent demonstration of the JH analogue-induced accumulation of Vg and another coordinately regulated protein in the haemolymph of *L. migratoria* is shown in Fig. 1.

In *L. migratoria*, a recent report indicates that Vg can be induced in last stage larvae and adults by ovary maturating parsin (Lo OMP), a neuropeptide from the brain, but ovariectomized larvae fail to respond, suggesting the participation of a factor from the ovaries, possibly (as in the Diptera) 20HE (Girardie and Girardie, 1996). In adult locusts, however, ovariectomy is followed by continuous accumulation of Vg to enormous levels, suggesting that the ovary may play a role in terminating Vg synthesis at the end of each gonotrophic cycle (Chinzei and Wyatt, 1985).

In the cricket, *Acheta domesticus* (Gryllidae), the normal appearance of Vg in the haemolymph of adult females at 16–24 h after emergence is prevented by neck ligation immediately after emergence; in neck-ligated animals the production of Vg and incorporation of radioactive methionine were induced by injection of JH I or JH III (Benford and Bradley, 1986). Ovarian development was also prevented by early application of precocene (Bradley and Haynes, 1991). In the Mediterranean field cricket, *Gryllus bimaculatus*, the appearance of Vg in the haemolymph was prevented by neck ligation or allatectomy and induced by methoprene; when normal oogenesis was inhibited by maintaining a low temperature, repeated application of 0.5 μg of JH III stimulated egg production but lowered the haemolymph Vg titre, presumably because of enhanced uptake into the oocytes (Kempa-Tomm *et al.*, 1990).

The stick insects (Phasmidae) present an exception to the generality of JH control of vitellogenesis among Orthoptera. In an early study on *Carausius morosus*, Pflugfelder (1937) reported that allatectomy in either the larval or early adult stage did not prevent production of viable eggs. From the data shown (Pflugfelder, 1958), however, it appears that egg maturation was significantly delayed after allatectomy. More recently, Bradley *et al.* (1989) have found that allatectomy had no effect on the rate of Vg synthesis and did not prevent the completion of egg development in *C. morosus*, but the uptake of Vg into the eggs was significantly retarded in the operated animals. It is of interest to note that in *C. morosus*, the terminal oocytes in the ovarioles undergo vitellogenesis with independent timing, and not in synchrony as in other Orthoptera, which would be consistent with a lack of endocrine coordination (Bradley and Giorgi, 1986). It thus appears that in this species the role of JH is reduced to a stimulatory influence on ovarian Vg uptake.

2.1.1.4 *Hemiptera.* It was in the blood-sucking bug, *Rhodnius prolixus* (Reduviidae), that Wigglesworth (1936), seeking an explanation for the

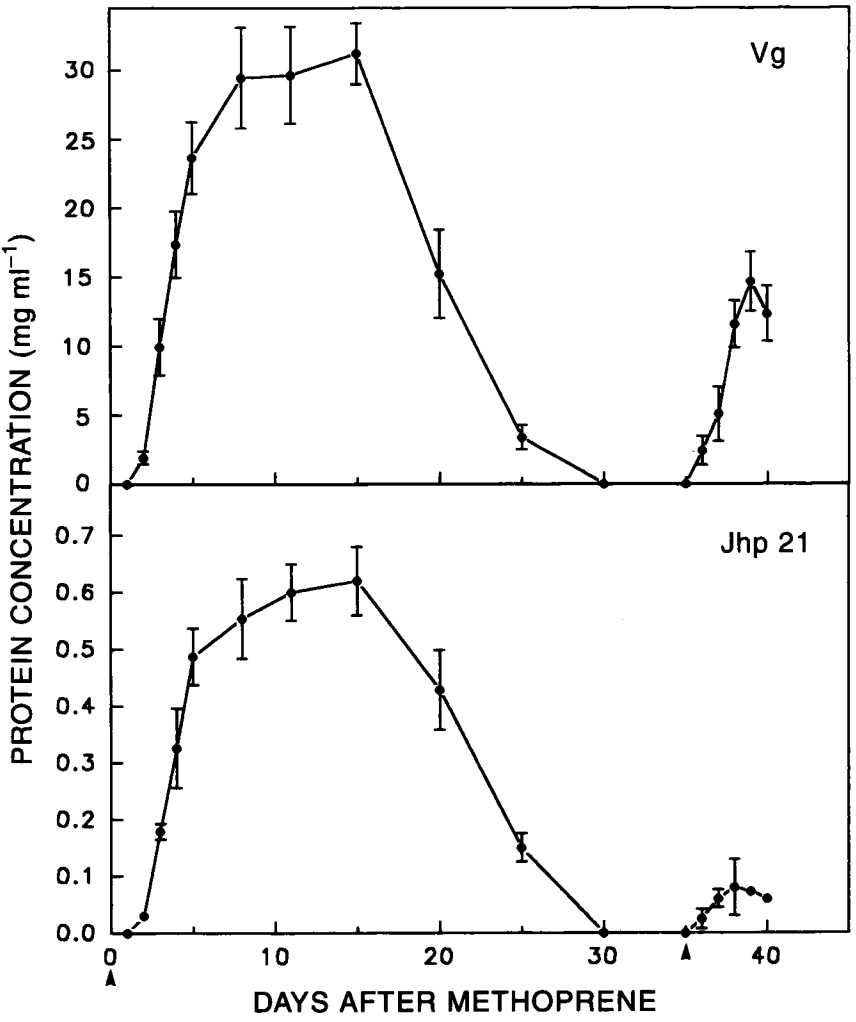

FIG. 1 Induction of Vg and Jhp21 protein in haemolymph of *Locusta migratoria* by methoprene. Adult female locusts, previously treated with precocene, were treated topically with 100 μg of methoprene in acetone on day 0 and again on day 35 (arrowheads). Haemolymph was sampled successively on the days indicated and analysed by rocket immunoelectrophoresis with antisera specific for Vg and Jhp21 protein, calibrated with purified proteins. Points show haemolymph concentrations as means ± S.E.M. (n = 10). The data show that the induction of the two proteins is coordinate in time, with secondary induction accelerated by 1 day relative to primary induction, and a much lower output of Jhp21 protein than of Vg (Zhang *et al.*, 1993).

production of eggs after a blood meal, first demonstrated a requirement for the CA. This species has continued to illuminate the interactions of feeding and mating in the regulation of oogenesis (review: Davey, 1993). A JH requirement for normal Vg production was demonstrated by allatectomy and replacement (Coles, 1964; Baehr, 1974). More detailed studies have shown, however, that *Rhodnius* females lacking CA can produce a low level of Vg and a limited number of eggs; administration of JH restores these processes to their normal rates, the synthesis of Vg in the fat body responding to a lower level of the hormone than the uptake into the ovarian follicles (Wang and Davey, 1993). This species is also unusual in that Vg appears in small amounts at the end of the fifth larval instar, and in the haemolymph of adult males after feeding, although at a lower level than in females (Chalaye, 1979). Synthesis in adult males is reported to be stimulated in a dose-dependent manner by methoprene (Chalaye and Lauverjat, 1985), although in another recent study a low dose of methoprene induced Vg synthesis in females but not in males (Chinzei *et al.*, 1994). cDNA for *R. prolixus* Vg has been cloned, which will facilitate studies on the control of its expression (Valle *et al.*, 1993). In another blood-sucking bug of the same family, *Triatoma protracta*, the Vg is adult- and female-specific, and its synthesis is apparently totally dependent on JH, since allatectomy at emergence prevented the appearance of this protein and synthesis was restored by administration of JH III (Mundall and Engelmann, 1977).

In the milkweed bug, *Oncopeltus fasciatus*, the dependence of vitellogenesis on the CA was demonstrated early (Johansson, 1954), but the subsequent literature describing effects on specific proteins is confusing. Assays using nondenaturing electrophoresis and immunodiffusion identified two female-specific components, one of which appeared not to be JH-dependent, whereas conversion to an 'antigenically complete' form, as well as uptake into the oocytes, were induced by JH (Kelly and Davenport, 1976; Kelly and Telfer, 1977; Kelly and Hunt, 1982). Another study could find no evidence for JH control of synthesis of Vg (Rankin and Jackle, 1980). More recently, the picture has been clarified by the use of SDS-PAGE (Martinez and Garcera, 1987). This technique revealed two female-specific Vg components of 200 kDa and 170 kDa, synthesis of which is prevented by treatment of newly emerged adults with precocene and induced by administration of JH I. They are found in the vitellogenic oocyte but not in the mature egg, in which they have been processed to smaller fragments. It is suggested that previous negative findings with respect to JH dependence may have been due to residual JH in diapause and starved bugs and to ineffectiveness of precocene when applied at too late a stage (Martinez and Garcera, 1987). Even after early precocene treatment, however, some Vg was produced 15–30 days later, and it is not certain whether this was due to residual or renewed JH production or to incomplete dependence of Vg expression on JH, as in *Rhodnius*.

Experiments with several other plant-feeding bugs have demonstrated dependence of Vg synthesis on JH. In the harlequin bug, *Dindymus versicolor*, both previtellogenic growth and maturation of the eggs were found to be controlled by the CA and JH (Friedel, 1974). In the red cotton bug, *Dysdercus intermedius*, allatectomy of adult females by decapitation prevented the appearance of female-specific polypeptides, and their production was restored by treatment with JH III (Dittmann *et al.*, 1985). In *D. koenigii* also, allatectomy prevented the appearance of Vg (Bhola *et al.*, 1988). In *Pyrrhocoris apterus*, the species used in early experiments that led to the identification of JH-active substances in certain wood products (Slama and Williams, 1965), a recent study showed that ability to produce vitellogenic polypeptides was abolished by allatectomy and restored by a JH analogue (4[(pivaloyl-L-alanyl)amino]benzoic acid ethyl ester); at the same time, the treatment induced the production of two other proteins of 78 and 82 kDa (Socha *et al.*, 1991). In the bean bug, *Riptortus clavatus*, application of methoprene to diapause females resulted in strong induction of synthesis of Vg, together with a cyanoprotein which is also deposited in the eggs (Chinzei *et al.*, 1992a, b).

In the bug, *Spilostethus pandurus*, three electrophoretic Vg bands (Ibanez *et al.*, 1992) appeared in the haemolymph and ovaries earlier in JH I treated adult females than in untreated controls, and were induced by JH when their production was inhibited by starvation (Ibanez *et al.*, 1993a, 1993b). Since endogenous JH was not eliminated from the controls, strict hormonal dependence of Vg synthesis cannot be concluded. The Vg bands did not appear in males after applications of JH to male larvae or adults (Ibanez *et al.*, 1990).

Thus, all of the species of Hemiptera that have been examined show stimulation of vitellogenesis by JH. In most instances, the dependence on JH appears to be complete, but in *Rhodnius* and *Oncopeltus* it may be leaky in that some delayed vitellogenesis can take place in the apparent absence of the hormone.

2.1.1.5 *Homoptera.* In this order, which is characterized by complex life cycles that include parthenogenesis, viviparity and polymorphism, a single experimental study has revealed some unusual adaptations. In the black bean aphid (*Aphis fabae*), the embryos of the next generation develop within the ovaries in fourth instar larvae, and growth of these embryos has been shown to be prevented by decapitation of the parent larvae and restored by administration of JH I (Hardie, 1987). It appears that the antimetamorphic role of JH is completed during the third (penultimate) larval instar, permitting the fourth instar to accommodate the gonadotrophic function of the hormone, which in other insects is normally deferred to the adult stage.

2.1.1.6 *Coleoptera.* There have been surprisingly few studies on the

hormonal control of vitellogenesis in beetles, the largest order of insects. In the Colorado potato beetle, *Leptinotarsa decemlineata*, adult activity depends on photoperiod, with long day-length bringing on reproductive activity, while short days induce diapause. When short-day beetles were treated with JH I or pyriproxyfen, Vg synthesis was induced (de Loof and de Wilde, 1970; Dortland, 1979; de Kort and Koopmanschap, 1992). Vg could also be induced in last instar larvae, where it does not normally occur, by application of pyriproxyfen (Koopmanschap *et al.*, 1992). However, allatectomy of long-day adults did not prevent Vg synthesis and egg development, whereas allatocardiacectomy was effective, which indicates that Vg synthesis can take place in the absence of JH, and additional control factors, presumably neuropeptide, must be involved. The ladybird beetle, *Coccinella septempunctata*, has been studied in China because of its use in biological control of aphids. When reared on an artificial diet, the beetles fail to reproduce and are deficient in vitellogenesis, but Vg synthesis can be induced by treatment with the JH analogue, ZR-512 (hydroprene) (Gong *et al.*, 1982; Zhai *et al.*, 1984, 1987; Guan, 1989); JH is, therefore, required for vitellogenesis and appears to serve as a signal of nutritional adequacy.

The *Vg* gene of the cotton boll weevil, *Anthonomis grandis*, has been cloned and completely sequenced (Trewitt *et al.*, 1992) but, as yet, the hormonal control of its expression has not been reported.

2.1.1.7 *Hymenoptera.* The Hymenoptera are of interest because of their diverse biology and great ecological importance, for example as parasitoids and as pollinators, but the control of vitellogenesis has been studied in only a few species. Wasps (*Polistes metricus*) collected in late summer were in reproductive diapause and did not develop eggs, but they could be induced to do so by application of JH (Bohm, 1972). In bumble bee (*Bombus terrestris*) workers, JH production and oogenesis were repressed by the presence of a queen in the colony, but oogenesis could be induced in them in a dose-dependent manner by JH I (Röseler, 1977); JH also induced Vg production in diapausing queens (Röseler and Röseler, 1988). In the sawfly, *Athalia rosae*, JH III applied to haploid and diploid males induced the appearance of Vg in the haemolymph (though less than its normal levels in females), and also stimulated the development of eggs in ovaries implanted into males; the role of JH in relation to vitellogenesis in females, however, was not examined (Hatakeyama and Oishi, 1990). The recent cloning of *A. rosae* Vg cDNA will facilitate further studies (Kageyama *et al.*, 1994).

In honey bees, which have a highly developed social structure, the situation is complex and still not fully understood. Eggs are normally produced only by the queen, which can synthesize more than 10 mg of Vg, or 20% of its dry mass, daily (Engels *et al.*, 1990). Vg is also present, however, at lower levels in the haemolymph of workers, which are sterile diploid females (Engels, 1972), and at still lower levels in drones (haploid males) (Trenczek and Engels, 1986).

Allatectomy of young workers prevented the appearance of Vg (Imboden *et al.*, 1976), but Vg and fertile eggs were produced in allatectomized queens after treatment with CO_2 (Engels and Ramamurthy, 1976). During the first 3 or 4 weeks of a worker's adult life, the activity of the CA and the haemolymph JH titre increase, while at the same time the haemolymph Vg content, after an initial rise, decreases; these changes are especially marked at about 20 days of age when the workers make the transition from hive duties (nursing and comb maintenance) to field duties (foraging) (Rutz *et al.*, 1976; Fluri *et al.*, 1982; Robinson *et al.*, 1989; Huang *et al.*, 1991). The haemolymph JH titres of foragers are far higher than those of queens, nurses and egg-laying workers from a queenless hive, which are similar to one another (Robinson *et al.*, 1991). The presence of the queen in a hive suppresses egg development in workers, and removal of the queen led to increased rates of JH biosynthesis in CA from young workers, which could be reversed by application of the queen pheromone, 9-oxo-2-decenoic acid (Kaatz *et al.*, 1992). However, when older, foraging workers switched to egg production as a result of removal of the queen, this was accompanied by reduced JH biosynthesis and lowered haemolymph JH titres (Robinson *et al.*, 1992). Results from the application of JH III or methoprene to workers indicate that Vg production and ovary development can be stimulated by low doses but suppressed by higher doses of the hormone, although a full dose–response relationship has not been determined (Rutz *et al.*, 1976; Robinson *et al.*, 1992). On the basis of such data, it is suggested that Vg synthesis may be stimulated in workers by the low, early levels of JH and later repressed by the higher JH titre at the transition from hive to field duties. JH may not be the controlling factor for Vg synthesis in this eusocial species, and there is evidence for participation of a neuropeptide from the brain (Engels *et al.*, 1990). The principal role of JH may be in regulating the sequence of duties of the workers (age polyethism; Section 2.5.3), which may have become possible as a result of the liberation of this hormone from responsibility for the control of vitellogenesis.

2.1.1.8 *Lepidoptera.* The Vgs were first characterized as 'female proteins' in the saturniid silkmoth, *Hyalophora cecropia* (Telfer, 1954) and, as it became clear that their synthesis in this species was independent of JH (Pan, 1977), it seemed that the Lepidoptera might be an exception to the general rule of JH regulation of Vg synthesis. In other species, however, vitellogenesis was found to be regulated by the CA, and it was suggested that JH regulation might be correlated with the ability of adults to feed (Wu and Quo, 1963; Sroka and Gilbert, 1971; Engelmann, 1983; Satyanarayana *et al.*, 1992; Cusson *et al.*, 1994b).

In the butterflies (Rhopalocera), where the adults feed and are relatively long-lived, egg maturation and Vg synthesis take place after adult eclosion and, in all species examined, are dependent on JH (review: Engelmann, 1983; *Pieris brassicae*: Kim *et al.*, 1988). In the monarch butterfly (*Danaus plexippus*;

Herman, 1975b; Pan and Wyatt, 1971, 1976) and the mourning cloak (*Nymphalis antiopa*; Herman and Bennett, 1975), reproductive development is related to photoperiod and temperature, and the brain-CA axis presumably conveys the signal. In *Danaus*, JH I and JH II were considerably more active than JH III (Lessman *et al.*, 1982).

Among moths, the Noctuidae are a large family which feed as adults and include many important agricultural pests. Control of vitellogenesis by JH has been found in all of the species examined. In the armyworm, *Leucania separata*, yolk deposition was prevented by decapitation of moths and restored by implantation of CA (Wu and Quo, 1963). In the corn earworm, *Helicoverpa zea*, egg production is blocked by allatectomy or decapitation and restored by implantation of CA or treatment with methoprene; the hormone may convey the signal of feeding and mating, both of which stimulate oogenesis (Satyanarayana *et al.*, 1991). In this species, decapitation immediately after adult eclosion did not completely prevent the appearance of Vg in the haemolymph unless preceded by a period of chilling at 10°C, which delayed vitellogenesis and permitted the induction of Vg synthesis by methoprene to be demonstrated (Satyanarayana *et al.*, 1992). Methoprene did not induce Vg in adult males, and 20HE had no effect. In the armyworm, *Pseudaletia unipuncta*, the timing of ovarian development is influenced by photoperiod and in short-day animals Vg production was completely prevented by decapitation after eclosion; induction of synthesis was then induced by topical application of JH or analogues (Cusson *et al.*, 1994b). JH I and JH II were about 10-fold more active than JH III, while methoprene and pyriproxyfen were about 25-fold and 300-fold, respectively, more active than JH I. In *Heliothis virescens*, too, JH I and JH II were more active than JH III in restoring egg maturation after decapitation (Ramaswamy and Cohen, 1991).

The tobacco hornworm, *Manduca sexta*, is a sphinx moth in which the adult feeds, and from several studies a somewhat complex picture has emerged. Vg first appears in the haemolymph and vitellogenesis in the oocytes commences in the late pharate adult, about 4 days before eclosion (Imboden and Law, 1983); these processes are not affected by allatectomy and are therefore independent of JH (Nijhout and Riddiford, 1974). The growth and maturation of eggs during the first days after eclosion, however, including additional Vg uptake, hydration and choriogenesis, require JH, since they are prevented by allatectomy or decapitation and are restored by implantation of CA or JH treatment (Sroka and Gilbert, 1971; Nijhout and Riddiford, 1974, 1979). The experiments cited were all performed with unfed, virgin females, in which egg maturation is arrested after day 3 of adult life. Further egg production and oviposition are stimulated by both feeding and mating (Sasaki and Riddiford, 1984; Ishizaka *et al.*, 1989). These stimuli appear to operate via the CA, since their effect can be mimicked by treatment with methoprene or, to some extent, JH II. It does not seem to be certain whether JH in the *Manduca* adult acts only, as assumed by

these authors, at the level of the ovary, since no data on its possible action on Vg synthesis in the fat body have been reported.

Recently, electrophoretic study of *M. sexta* haemolymph has revealed low levels of Vg in prepupae (days 6 and 7 of instar V) of both sexes, which disappeared by the pupal ecdysis, to reappear, only in females, in the late pharate adult, as described above (Satyanarayana *et al.*, 1994). Vg could be further induced in prepupae or day 0 pupae by treatment with methoprene; females responded more strongly than males, and the response was blocked by simultaneous injection of 20-hydroxyecdysone (20HE). Induction of Vg synthesis was accompanied by the presence of a 5.1 kb band in fat body RNA. It is not clear that this early appearance of Vg has any significance for the economy of the hornworm, but the inducibility by a JH analogue and the lack of strict sex-limitation at this stage are of interest. The authors suggest that the Vg genes become expressible after the 'commitment' 20HE peak on day 4 of instar V, are expressed under the ensuing prepupal peak of JH, and then during the major ecdysone peak in the early pharate adult undergo a transition to a JH-independent (but female-limited) state which leads to their expression without JH in the late pharate adult (Satyanarayana *et al.*, 1994). It will be interesting to determine whether the further Vg synthesis that takes place after eclosion, as discussed above, is JH-responsive.

In several moth species where the adult is short-lived and does not feed, Vg is produced before eclosion, chiefly in the pharate adult, independently of JH action. This was first shown by allatectomy of larval instars of the oriental silkworm, *Bombyx mori*, which resulted in premature metamorphosis to miniature adults that produced eggs containing Vg (Bounhiol, 1938; Fukuda, 1944; Lamy *et al.*, 1975). In the saturniid silkmoth, *Hyalophora cecropia*, it has been clearly shown that the appearance of Vg in the haemolymph and incorporation of [^{3}H]leucine were unaffected by allatectomy or administration of JH I (Pan, 1977). In the tent caterpillar, *Malacosoma pluviale*, allatectomized pupae developed to produce normal numbers of eggs (Sahota, 1969). In the Indianmeal moth, *Plodia interpunctella*, treatment of pharate adults with methoprene failed to reverse inhibition of ovary development brought about with ecdysone (Shirk *et al.*, 1990). However, in another pyralid moth, *Ephestia cautella*, oviposition is stimulated by mating and the number of eggs laid by virgin females is enhanced after application of methoprene (Shaaya *et al.*, 1991); a similar effect has been reported for the tortricid, *Platynota stultana* (Webster and Cardé, 1984).

The gypsy moth, *Lymantria dispar*, is unique among insects so far reported in that Vg appears in the haemolymph of females as early as day 3 of the final larval instar, even though ovarian vitellogenesis does not commence until some 13 days later, on day 3 of the pupal stage (Davis *et al.*, 1990; Lamison *et al.*, 1991). Treatment of larvae on day 2 of the last instar with methoprene or fenoxycarb selectively prevents the appearance of Vg in the haemolymph, and that of Vg mRNA in the fat body (Davis *et al.*, 1990;

Hiremath and Jones, 1992; Kelly *et al.*, 1992). This repression of Vg genes by JH analogues is unusual but, occurring in the early last-instar larva, it fits the context of the negative regulation of many genes by JH in premetamorphic insects (review: Riddiford, 1994). Gypsy moth Vg cDNA has been cloned, which will facilitate molecular studies (Hiremath *et al.*, 1994; Adamczyk *et al.*, 1996). JH is apparently required for oviposition in gypsy moths, since moths that developed from larvae allatectomized in the late last instar failed to deposit eggs (Wang and Yin, 1983).

2.1.1.9 *Diptera.* Certain mosquitoes, because of their importance as disease vectors, have been the object of intensive investigation of the endocrine regulation of oogenesis. Most of the published work over some 30 years has been concentrated on *Aedes aegypti*, an anautogenous species (requiring a blood meal to initiate oogenesis), which is the vector of yellow fever and is amenable to laboratory maintenance. A smaller amount has been done on other species. The mosquitoes, which represent the more primitive suborder Nematocera, utilize Vgs homologous with those of other animals, whereas the more advanced suborder Cyclorrhapha (including muscoid flies and fruit flies) have abandoned conventional Vgs and adopted a distinct family of proteins for their egg yolk reserves (see Section 2.1.2).

The research on mosquito vitellogenesis has been characterized by unexpected discoveries, controversy, and the development of an increasingly complex model of regulation involving neuropeptides, JH and ecdysteroids, which differs from the scheme for other insect groups. The work has been thoroughly reviewed by the protagonists (Fuchs and Kang, 1981; Hagedorn, 1983, 1985, 1989; Raikhel, 1992; Dhadialla and Raikhel, 1994). The fat body is apparently the sole site of Vg synthesis (in contrast to the yolk proteins of higher Diptera) and we shall focus here on the roles of JH in stimulating fat body activity.

In *A. aegypti*, the first cycle of egg maturation is divided into a previtellogenic period of 3–5 days after eclosion of the adult female, leading to a resting period or state of arrest, followed by a vitellogenic period which is initiated by a blood meal and lasts about 48 h for completion of oogenesis. The most novel element of hormonal control, relative to the accepted picture for other insect orders, is the importance of 20HE as the immediate inducer for Vg synthesis. Spielman *et al.* (1971) first reported that mosquitoes fed 20HE could mature eggs without a blood meal. Hagedorn and co-workers confirmed the effect of 20HE and assembled evidence supporting the following model: the blood meal causes release from the brain of a neurohormone (EDNH = egg development neurosecretory hormone) which acts upon the ovary to cause it to produce ecdysone; this passes to the fat body where it is converted to 20HE and induces synthesis of Vg; the Vg is transported to the ovary, taken up and deposited in the yolk as in other insects (reviews: Hagedorn, 1983, 1985). This scheme, which was based upon

experiments both *in vivo* and with tissues incubated *in vitro*, received criticism (Borovsky and Van Handel, 1979; Fuchs and Kang, 1981) chiefly on the grounds that the dose of 20HE required to induce vitellogenesis in non-blood-fed mosquitoes was several thousand times greater than expected from physiological considerations, and the yield of Vg was only a few per cent of that produced after a blood meal. The data were also difficult to reconcile with the fact that in the autogenous species, *A. atropalpus*, physiological doses of 20HE were sufficient to induce Vg synthesis (Kelly *et al.*, 1981). While the poor response in *A. aegypti* was explained partly in terms of degradation of the administered 20HE and lack of nutrient reserves in the non-blood-fed females (Hagedorn, 1983), and possible inhibitory factors *in vivo* (Raikhel, 1992), a further essential factor proved to be that the fat body be in a state of full competence to respond to ecdysone and produce Vg. It is here that JH is involved.

A requirement for the CA in *A. aegypti* egg development was first indicated by Larson and Bodenstein (1959), and Lea (1969) showed by allatectomy that these glands were required during the previtellogenic phase but not after the blood meal. JH is released from the CA after adult emergence (Gwadz and Spielman, 1973), leading to a high endogenous JH titre which declines after the blood meal (Shapiro *et al.*, 1986). The competence of fat bodies cultured with 20HE to respond by synthesis of Vg was shown to develop during the first days after eclosion, and this process was dependent on the presence of the CA or administration of JH (Flanagan and Hagedorn, 1977; Ma *et al.*, 1988). In *A. atropalpus*, too, application of a small dose of JH to abdomens isolated shortly after emergence greatly enhanced their response to a subsequent injection of 20HE (Kelly *et al.*, 1981).

JH also appears to play a role in *A. aegypti* fat body after the blood meal, although this is less clearly established. Borovsky (1981) reported that mosquitoes decapitated, or abdomens ligated, immediately after feeding, could develop yolk-filled eggs in response to nanogram doses of methoprene or JH I alone. A smaller dose (25 pg) of methoprene did not induce vitellogenesis, but when this was followed by 5 ng of 20HE egg development with vitellogenesis took place (Borovsky *et al.*, 1985). This indicated that exposure to JH soon after the blood meal could potentiate vitellogenesis in response to physiological levels of 20HE; the previously noted egg development in response to higher doses of JH or analogue alone might be due to the stimulation of ovarian ecdysone synthesis by the pharmacological level of JH. Further experiments with single and double doses of hormones showed that a low dose of either methoprene or 20HE applied to abdomens isolated immediately after the blood meal resulted in greatly increased sensitivity to 20HE administered 18 h later (Martinez and Hagedorn, 1987); from these experiments it was uncertain which hormone served as the endogenous primer. However, in fat bodies incubated *in vitro*, the presence of 10^{-5} M methoprene enhanced the production of Vg mRNA, assayed with a cloned

DNA probe (Racioppi *et al.*, 1986). The inference that, early after the blood meal, JH may be priming the fat body for Vg production in response to 20HE appeared to conflict with the earlier demonstration that the CA were dispensable during this phase of the reproductive cycle (Lea, 1969); however, this paradox may be resolved by the recent report that JH III can be synthesized in the ovaries themselves of *A. aegypti* (Borovsky *et al.*, 1994a). There is also recent evidence for a synergistic effect of a factor released from the midgut after feeding (Dhadialla and Raikhel, 1994).

The current view, then, in this complex and controversial story is that JH is required in the previtellogenic period in order to bring the fat body to competence for response to ecdysteroid and synthesis of Vg, a process that we describe as priming (Section 3.3). Key cellular events appear to include increase in ploidy (Dittman *et al.*, 1989), proliferation of ribosomes (Hotchkin and Fallon, 1987; Raikhel and Lea, 1990) and production of ecdysone receptors (Cho *et al.*, 1995), as will be further discussed under Section 3.5. In the vitellogenic period, after the blood meal, 20HE appears to be the principal regulator in inducing fat body Vg synthesis, while JH, along with other factors, may contribute further to enhancing the response of the fat body early in this phase.

2.1.1.10 *Resumé.* The involvement of JH in the regulation of Vg synthesis in sample insects belonging to eight orders is summarized in Table 1. Some interpretive generalizations can be attempted. The regulation of Vg synthesis by JH appears to be an ancient system in insects and is found generally in the exopterygote orders, with a few exceptions: little or no control in a phasmid, and apparently incomplete control in some Hemiptera. Among the endopterygote orders, control by JH is still usual, but more adaptations to specialized ways of life have evolved. Thus, in the honey bee worker the major role of JH appears to be in the expression of sequential behaviour patterns, and the honey bee queen, which produces eggs continuously for months on end, has escaped the need for JH regulation. Among the Lepidoptera, those species or groups in which eggs are matured in the adult in response to stimuli such as feeding, mating and photoperiod make use of JH to transduce these signals, whereas those in which oogenesis is completed before adult eclosion do this without JH regulation, as an integral part of adult organogenesis. The nematocerous Diptera – so far as one can generalize from studies focused on one species of mosquito – represent a special case in which JH-supported differentiation is a prerequisite for further hormonal response and yolk protein synthesis, but the immediate control function has been taken over by 20HE.

2.1.2 *Yolk proteins of the higher diptera*

The process of vitellogenesis in higher Diptera (Cyclorrhapha) differs from that in other insects in a number of significant ways. Those proteins that are

TABLE 1 Effects of JH on Vg synthesis in different insect groups

Order, insects	Stage of Vg synthesis	Effect of JH
Thysanura		
Firebrat	Adult	Induction, coordinated with moulting
Dictyoptera		
Cockroaches	Adult	Induction
Orthoptera		
Locusts, grasshoppers, crickets	Adult	Induction
Stick insect	Adult	Little or no effect
Hemiptera		
Several bugs	Adult	Stimulation or induction
Coleoptera		
Beetles (2 species)	Adult	Stimulation or induction
Lepidoptera		
Gypsy moth	Last stage larva	Repression
Silkmoths	Pharate adult	No effect
Cutworms, armyworms	Adult	Induction
Butterflies	Adult	Induction
Hymenoptera		
Wasp, bumble bee, sawfly	Adult	Induction
Honey bee worker	Adult	Induction and repression
Honey bee queen	Adult	No effect?
Diptera		
Mosquitoes	Adult	Priming for 20HE regulation

This tabulation is based on studies in which Vg protein was assayed. Induction signifies that synthesis is dependent on JH; stimulation signifies that synthesis can occur without JH, but is enhanced by JH. For scientific names and references, see text.

synthesized in the fat body and sequestered by the oocyte are unlike the Vgs of other insects. They are best known from *Drosophila melanogaster* in which three subunits, YP1, YP2 and YP3 of 46, 45 and 44 kDa respectively have been identified (Harnish and White, 1982). These subunits, unlike those of the Vgs of other insects, are not the products of proteolytic processing, but are derived from the primary translation products by the addition of phosphate, sulfate and carbohydrate (Brennan *et al.*, 1982; Brennan and Mahowald, 1982; Minoo and Postlethwait, 1985; Di Mario *et al.*, 1987). Other species of *Drosophila* have similar yolk proteins (Srdić *et al.*, 1978; Postlethwait, 1980), and yolk proteins of similar size have been described in various muscoid genera, including houseflies (Adams and Filipi, 1983), blowflies (Jensen *et al.*, 1981; Fourney *et al.*, 1982; Huybrechts and de Loof, 1982), and fleshflies (Huybrechts and de Loof, 1982), as well as in various tephritoid genera of fruit flies (Levedakou and Sekeris, 1987; Rina and Mintzas, 1987; Handler and Shirk, 1988).

Not only are these yolk proteins composed of subunits of a different size from those of the Vgs, but sequence analysis demonstrates that they are not homologous to the Vgs, and show some homology to mammalian lipase (Bownes *et al.*, 1988; Terpstra and AB, 1988). Because of these clear structural differences from the Vgs, the yolk proteins of the higher Diptera have been designated as 'yolk proteins' or 'yolk polypeptides' (YP).

Furthermore, the YP in higher Diptera are not synthesized exclusively in the fat body: to a greater or lesser degree the ovaries also participate in the synthesis. Both tissues participate in the synthesis of YP in *Drosophila* and some other species (Srdić *et al.*, 1979; Gutzeit, 1980; Bownes, 1982), but in the stable fly, *Stomoxys calcitrans* (Chen *et al.*, 1987), and possibly the tsetse fly, *Glossina austeni* (Huebner *et al.*, 1975), they are produced exclusively by the ovary. In the *Drosophila* ovary, it is the follicle cells which synthesize the YP. Based on mRNA analysis, the ovary in *Drosophila* produces about 35% of YP1 and YP2, but only about 12% of YP3 (Brennan *et al.*, 1982).

The hormonal control of the synthesis of these proteins also presents a complex picture. While there are species differences, and there are still areas of uncertainty, the most complete description of the endocrine control of YP synthesis in higher Diptera is provided by *Drosophila*. It is important to recognize that the synthesis of YP is initiated before eclosion of the fly from the puparium (Kambysellis, 1977), and, as a consequence, classical ablation/replacement studies on adults may identify those hormone sources important for the maintenance of egg development but may not be relevant to its initiation.

In *Drosophila* decapitated before eclosion, at a time before synthesis of YP can be detected, no vitellogenesis is subsequently detected. But if such flies are treated with methoprene, normal vitellogenesis ensues (Handler and Postlethwait, 1978), and the interruption of vitellogenesis which occurs when females are decapitated after eclosion can be reversed by methoprene, or, less effectively, by implanting a CA. Carefully timed decapitations indicated that a factor from the head also plays a role in the control of vitellogenesis (Handler and Postlethwait, 1978).

Building on these pioneering studies, several authors (see Kelly, 1994) have shown that ecdysone acts on the fat body to stimulate YP synthesis, and, while there are several important gaps in the evidence, the evidence thus far is consistent with the model proposed by Hagedorn (1985) for the control of vitellogenesis in mosquitoes. That is, a factor from the brain similar in action to the egg development neurosecretory hormone of mosquitoes causes the synthesis and release of ecdysone from the ovary. The ecdysone acts on the fat body to initiate and sustain YP synthesis (Kelly, 1994).

The model for *Drosophila*, however, deviates from the mosquito model in some significant ways. First, of course, both the follicle cells and the fat

body manufacture the YP in *Drosophila*, while only the fat body is responsible for Vg synthesis in mosquitoes. Second, females of the *tudor* mutant, in which ovaries are absent, exhibit normal titres of ecdysteroids two days after eclosion, the titre dropping on the third day (Bownes, 1989). This suggests either some carry-over of ecdysone from the pupa, or an alternative source of the hormone in the adult. Experiments conducted by Jowett and Postlethwait (1980) bring out some additional differences. They isolated the abdomens of females immediately after eclosion and 24 hours later injected either JH or ecdysone and measured the incorporation of methionine into YP by tissues *in vitro*. Either JH or 20HE appeared to be capable of stimulating YP synthesis in the fat body (the fat body preparation included the body wall). Injection of JH stimulated YP synthesis in the ovaries, while injection of 20HE alone was without effect. Because these experiments do not eliminate the possibility that the isolated abdomens contain a source of ecdysteroid, interpretation is a little difficult, but they do demonstrate that 20HE alone will not support YP synthesis by the ovary. Similar experiments using monoclonal antibodies to detect YP showed that no YP was found in female *Drosophila* decapitated immediately after eclosion, and that a single application of JH or methoprene restored YP production. Treatment of fat body *in vitro* with 20HE stimulated YP production (Wu *et al.*, 1987).

These, and other experiments on *Drosophila*, leave unanswered the question of a requirement for JH in order to prepare the fat body to respond to ecdysone, as occurs in mosquitoes (Section 2.1.1.9). Observations on blowflies, however, suggest that such a mechanism is operating. It is first of all clear that ecdysone is responsible for controlling YP synthesis in the fat body of blowflies. Females from four genera (*Lucilia*, *Phormia*, *Sarcophaga* and *Calliphora*) all behave similarly. Sugar-fed females do not produce YP. Injection or feeding of ecdysone supports the production of YP whereas injection or feeding of methoprene does not (Huybrechts and De Loof, 1982). Thomsen and Thomsen (1974) had earlier shown that fat body from ovariectomized females of *Calliphora erythrocephala* did not contain the granules associated with YP after a protein meal, although the tissue developed the synthetic machinery characteristic of vitellogenic females. Injection of ecdysone resulted in the appearance of the secretory granules. Allatectomy of *Phormia regina* before they had taken a protein meal prevented vitellogenesis, whereas allatectomy after a protein meal did not. The fat body in these flies did not appear to respond to JH by synthesizing YP. Taken together, these facts conform to the view that JH prepares the fat body to respond to ecdysone from the ovaries, as has been more clearly and crisply demonstrated for mosquitoes. By allatectomizing adult females of the blowfly, *Phormia regina*, and treating with methoprene at various times after a liver meal, Qin *et al.* (1995) have further explored

the role of JH in the control of vitellogenesis. The results support the general model for Diptera, in which JH: primes the fat body to respond to 20HE; primes the follicle cells to respond to egg development neurosecretory hormone by synthesizing ecdysone; enhances the production of ecdysone by the follicle cells; and regulates patency in the follicular epithelium. Interestingly, they demonstrate a low level of YP in flies lacking both the CA and ovaries, and suggest that the YP genes may be weakly expressed in the absence of hormonal stimulation.

Houseflies also exhibit a dual control over YP synthesis and vitellogenesis (Adams and Filipi, 1988). In flies that had the entire retrocerebral complex removed immediately after eclosion, the levels of YP in the haemolymph remained low, but were restored to normal by treatment with methoprene. Ecdysone, by contrast, had no effect. Flies that were ovariectomized exhibited a slow increase in YP levels, which was enhanced by ecdysone but not JH. Flies lacking both the retrocerebral complex and ovaries had undetectable levels of YP in the haemolymph, and methoprene restored the control levels, while ecdysone had a lesser effect. The two hormones could act synergistically, so that smaller simultaneous doses restored YP levels. These results are consistent with a model in which the fat body synthesizes the YP in response to either JH or ecdysone, but a prior exposure to JH is required in order for ecdysone to have its effect.

The discovery that the CA of larvae of *Drosophila* (Richard *et al.*, 1989) and the adults of *Calliphora vomitoria* (Cusson *et al.*, 1991; Duve *et al.*, 1992) are capable of secreting the 6,7-epoxide of JH III (JHB_3) as their principal product is a further potential complication. Much of the earlier experimental work has been based on the reasonable asssumption that JH III is the active form of the hormone, and it is thus far uncertain what relationship may exist between the activity of JH III and that of its bisepoxide. Moreover, many of the hormone replacement studies have been conducted using methoprene as a JH substitute, and the relationship of this analogue to JHB_3 is also uncertain. In *P. regina*, isolated CC-CA *in vitro* synthesize, in descending order of quantity, JHB_3, JH III and methyl farnesoate (MF) (Yin *et al.*, 1995). All three juvenoids can restore egg production in flies deprived of their CA, in the order of potency $JHB_3 > JH\ III > MF$. Perhaps significantly, a much lower dose of the blend of all three compounds in the ratio in which they are produced is required to restore egg production than other ratios or any one of the juvenoids alone. This raises the possibility that there may be separate receptors for each of the hormones.

An obvious lacuna in our knowledge of the reproduction of the Diptera is the total lack of information about the structure and regulation of yolk proteins of the suborder Brachycera, a group in some respects intermediate between the Nematocera and the Cyclorrhapha that includes the horse flies. The examination of one or more species from this group would form a significant contribution.

2.1.3 *Other yolk components*

Several other classes of protein have been identified as being produced chiefly in the fat body of certain insects, secreted into the haemolymph, and taken up selectively into the growing oocytes (Telfer and Pan, 1988). Some of these are influenced by JH.

2.1.3.1 *Microvitellogenins.* A low molecular weight (about 31 kDa), female-specific protein, first observed in the silkmoth, *Hyalophora cecropia*, as a minor component of haemolymph and yolk, was designated microvitellogenin (Telfer and Kulakovsky, 1984). A similar protein in *Manduca sexta* is synthesized from the early pupa into the adult stage (Wang *et al.*, 1989); decapitation of late pharate adults to eliminate JH did not affect its synthesis but did reduce uptake into the eggs (Kawooya and Law, 1983; Kawooya *et al.*, 1986). It will be of interest to examine other lepidopteran species, in which the synthesis of Vg is regulated by JH, for possible influence on the synthesis of their microvitellogenins.

2.1.3.2 *Silkworm 30K proteins.* The silkworm, *Bombyx mori*, is unusual in that the eggs contain, in amounts almost equal to vitellin, a 30 kDa protein ('30K protein') which is produced in the fat body and taken up from the haemolymph (Zhu *et al.*, 1986). cDNA sequence analysis indicates a small family of closely related proteins (Sakai *et al.*, 1988), one of which has a nucleotide sequence 70% identical to that of *M. sexta* microvitellogenin (Wang *et al.*, 1989). In *B. mori*, the 30K proteins are not sex-specific and in late last instar larvae they are the major haemolymph proteins; their appearance at this time coincides with the disappearance of JH (Izumi *et al.*, 1981), and, at an earlier stage, allatectomy brings on their synthesis (Izumi *et al.*, 1984). Thus, expression of the 30K protein genes in the larva is repressed by JH, typical of JH effects in the premetamorphic stage. Their expression in the adult has not been reported.

2.1.3.3 *Locust protein Jhp21.* In *Locusta migratoria*, a 21 kDa protein (Jhp21) has been characterized which is female-specific, synthesized in the fat body, dependent on JH for induction in adults or (with high doses of JH analogue) in last instar larvae, and taken up into vitellogenic oocytes to become a minor component of yolk (Zhang *et al.*, 1993; Zhang and Wyatt, 1996). The *jhp21* gene in the locust is therefore JH-regulated and is expressed coordinately with the *Vg* genes, although at a much lower level. The sequence from the cloned cDNA shows no homologies with other known proteins, except for a short N-terminal identity with the locust 19K protein (Section 2.1.4.2), and the function of the Jhp21 protein is not known. It does not appear to be related to the 19 kDa JH-regulated egg calmodulin of a cockroach (Zhang and Kunkel, 1992; Iyengar and Kunkel, 1995). The locust

jhp21 gene has recently been cloned with extensive upstream DNA, and the control of its transcription is being studied (Section 3.5.4).

2.1.3.4 *Bean bug cyanoprotein.* The bean bug, *Riptortus clavatus*, produces a blue cyanoprotein which is a hexamer of 76 kDa subunits that bind biliverdin (Chinzei *et al.*, 1990, 1991a). The amino acid composition, rich in tyrosine and phenylalanine, and the N-terminal sequence indicate a relationship to the arylphorins and other hexamerins (Telfer and Kunkel, 1991; Miura *et al.*, 1994). This protein is unusual among the described insect cyanoproteins since it is present in both larval and adult haemolymph of both sexes, and is taken up into the oocytes, to form about one-third of the protein of mature eggs. The subunits are of two kinds, *a* and *b*, that associate in varying proportions, and only the a_6 form of the protein (CP-1) accumulates in the eggs, whereas the b_6 form (CP-4) and the forms containing both subunits are found only in the haemolymph (Chinzei *et al.*, 1991b). Under short-day conditions, adult bugs remain in diapause, and haemolymph of both sexes contains CP-1, CP-4 and the heterohexamers. When diapause is broken, however, either by long-day exposure or by treating short-day insects with a JH analogue such as methoprene, a response is observed that differs interestingly in the two sexes: in females CP-1 is actively produced in the fat body to build up in the haemolymph and accumulate in the growing eggs, while CP-4 changes little in the haemolymph (and does not enter the eggs); in males synthesis of both CP-1 and CP-4 ceases and after 7 days they are undetectable in the haemolymph (Miura *et al.*, 1991). In adult bean bugs, synthesis of the two cyanoprotein subunits is regulated by JH differentially according to sex: in females subunit *a* (CP-1) is strongly induced and subunit *b* (CP-4) is repressed by the hormone (although this repression may be followed at later times by a weak induction), while in males both subunits are repressed (Chinzei *et al.*, 1992a, b). These effects are seen in the mRNAs, and so presumably reflect transcriptional regulation (Miura *et al.*, 1992). CP-1 may serve as an egg yolk protein, but it is not known what functions the CPs may exercise in males and diapause females. The differential control of the two subunits provides an opportunity for analysis of mechanisms of JH regulation.

2.1.4 *Other haemolymph proteins*

Studies on diverse insects have shown that haemolymph proteins of several other types are subject to regulation by juvenile hormone.

2.1.4.1 *Hexameric storage proteins.* Many of the major haemolymph proteins of insects belong to a superfamily that comprises hexamers of about 75–80 kDa subunits, with sequences that show homology with the arthropod haemocyanins (Telfer and Kunkel, 1991; Beintema *et al.*, 1994). Most of those described are 'storage proteins' that accumulate in the haemolymph in the last

larval instar and serve to provide amino acids for the construction of the adult integument and other organs in metamorphosis (Kanost *et al.*, 1990). The synthesis of several of these in last-instar larvae is known to be repressed by JH (Jones *et al.*, 1993a, b; Riddiford, 1994). Some other hexamerins, found in both larval and adult stages, have adopted more specialized roles such as the binding of biliverdin or JH (Sections 2.1.3.4, 3.1.3).

Several insects possess storage hexamerins that persist into the adult stage and are subject to JH regulation. In *Locusta*, a 'persistent storage protein' (PSP) (Ancsin and Wyatt, 1990; Wyatt, 1990; Ancsin and Wyatt, 1996) is abundant in haemolymph of the late last-instar larva, at which time its accumulation can be prevented by administration of a JH analogue, like that of several lepidopteran storage proteins. In the early adult locust, PSP declines to a low level, but later during JH-induced reproductive maturation its concentration rises, coincident with the beginning of Vg synthesis, to make it a major haemolymph component (Wyatt, 1990; Wyatt *et al.*, 1992b). In JH-deficient (precocene-treated) adults, PSP maintains a low constitutive rate of synthesis, which is strongly elevated by JH or an analogue. The direction of the JH effect upon PSP synthesis in the locust fat body is therefore switched during metamorphosis from repression to stimulation.

In female adults of the cockroach, *Blaberus discoidalis*, decapitation, which eliminates JH and prevents Vg synthesis, also caused an unexpected strong induction of synthesis of two other polypeptides (Bradfield *et al.*, 1990). The effect was seen as a doublet of 2.4 kb mRNAs, which disappeared after application of methoprene *in vivo* or to fat body *in vitro*. One protein was identified by cDNA sequence as a hexamerin with 86 kDa subunits rich in tyrosine and phenylalanine (Jamroz *et al.*, 1996). Its mRNA is abundant in the fat body of adult female cockroaches on the day of emergence and normally declines sharply by day 3, to persist at a detectable but very low level. After allactectomy on day 0, however, expression continued, and could then be repressed by repeated treatment with JH III. The second 2.4 kb mRNA belonged to a transferrin (Section 2.1.4.4).

In *Rhodnius prolixus*, a hexameric storage protein is found in both larval and adult haemolymph, and its synthesis in adults of both sexes was stimulated after engorgement and after application of methoprene (Chinzei *et al.*, 1994).

In the Colorado potato beetle, *Leptinotarsa decemlineata*, diapause protein 1 is a hexamerin with 82 kDa subunits which occurs in the haemolymph of last-instar larvae and short-day adults in reproductive diapause, stages which are deficient in JH, but is absent in reproductively active long-day adults. It probably provides an amino acid reserve for construction of flight muscles and other adult structures. When a low dose of pyriproxyfen was applied to either larvae or short-day adults, levels of the protein were depressed and its specific mRNA disappeared within a few days (de Kort and Koopmanschap, 1992, 1994; Koopmanschap *et al.*, 1992).

Thus, three storage hexamerins in different insect species show distinct patterns of regulation by JH. In last-stage larvae two at least are repressed by JH but in adults two of them – the *Blaberus* storage protein and the Colorado beetle diapause protein – are repressed, while the locust PSP shows a reversed response and is induced by the hormone.

2.1.4.2 *Locust 19K protein.* In *L. migratoria* at about day 8 of adult life, when Vg first appears and synthesis of persistent storage protein rises in response to rising JH titre, there is also an accumulation of a small haemolymph protein (Wyatt, 1990; Wyatt *et al.*, 1992). This has been identified as a 19 kDa protein and its cDNA has been cloned (Kanost *et al.*, 1988). It shows N-terminal sequence similarity to the locust ovary-destined Jhp21 protein (Section 2.1.3.3) and some internal similarity to *Torpedo* acetylcholinesterase and bovine thyroglobulin, but its function is unknown. Unlike the Jhp21 protein, the 19K protein is not sex-specific. Its mRNA level is high in the newly emerged adult and low from days 2 to 6, followed by a rise; it is depressed (but not abolished) after precocene treatment and elevated after application of methoprene (Kanost *et al.*, 1988). Thus, the 19K protein, like the persistent storage protein, is produced constitutively but enhanced by JH.

2.1.4.3 *Cold-hardiness and desiccation proteins.* Different insects make use of several mechanisms to avoid the potentially lethal effects of extreme winter cold (Zachariassen, 1985). These include lowering the supercooling point by production of antifreeze proteins (as well as low molecular weight solutes such as polyols) and, in some instances, removal of lipoprotein ice nucleators from the haemolymph; in both processes JH appears to be involved. In larvae of the beetle, *Dendroides canadensis*, treatment with precocene prevented the elevation of thermal hysteresis of the haemolymph (a measure of its antifreeze protein content) under normally inducing photoperiod and temperature, whereas prolonged treatment with JH I under normally non-inducing conditions caused an increase in this parameter (Horwath and Duman, 1983). Similarly, JH treatment of larvae of the stag beetle, *Ceruchus piceus*, has been reported to cause a decrease in supercooling points and in haemolymph ice-nucleator lipoprotein activity, although, since the protein itself did not disappear, the induction of a modified, inactive form was suggested (Xu *et al.*, 1990). In early pupae of the mealworm, *Tenebrio molitor*, methoprene treatment caused enhanced synthesis of a desiccation stress protein (dsp28), which is also inducible by low temperature (Kroeker and Walker, 1991).

In contrast to these premetamorphic effects are observations on the bug, *Pyrrhocoris apterus*, which overwinters as freezing-sensitive adults (Hodkova *et al.*, 1992). After larval rearing under short-day conditions, *P. apterus* adults had low supercooling points regardless of short-day/low temperature acclima-

tion in the adult stage, whereas after larval rearing under long days, adult acclimation led to lowered supercooling point only in allatectomized individuals, so that the presence of JH seemed able to prevent supercooling point lowering. Thus, cold-resistance proteins appear to be induced by JH in certain insects before metamorphosis, and repressed in others after metamorphosis, reminiscent of other examples of role-reversal for JH during this transformation.

2.1.4.4 *Cockroach transferrin.* A second protein repressed by JH in adult *Blaberus discoidalis*, along with a hexameric storage protein (Section 2.1.4.1), was identified by cDNA cloning as the iron transport protein, transferrin, homologous with vertebrate transferrins. The cockroach transferrin mRNA, similarly to that of the storage protein, is suppressed in adult females by JH III (Jamroz *et al.*, 1993; Fig. 9). The biological significance of this striking regulatory mechanism is unknown.

2.2 GONADS

2.2.1 *Ovaries*

In some insects, all of the eggs are produced in the pupal stage, but in many species at least some of the egg production occurs in the adult. There are several phases in the production of an egg. The oogonia multiply by mitosis, and in meroistic ovaries some of the oogonia become specialized as nurse cells (trophocytes). In the telotrophic ovary, the nurse cells may retain their ability to divide by mitosis after the oocytes have differentiated. In most insects, meiosis is delayed until the spermatozoon enters the oocyte, and the nucleus of the unfertilized egg is the typical germinal vesicle, ready to enter the prophase of the first maturation division. As the oocytes, and, in polytrophic ovaries, their accompanying nurse cells, descend in the ovariole, they pass through an area of proliferating cells, the follicular primordium, and accumulate a layer of follicle cells around themselves. The follicle enters upon a phase of previtellogenic growth characterized by intra-follicular synthesis. This is followed by vitellogenic growth, in which the oocyte accumulates yolk. Typically, the principal yolk component is a protein, vitellin, which is synthesized by the fat body as Vg (Section 2.1.1) and released into the haemolymph. The Vg passes between the cells of the follicular epithelium (Telfer, 1961), to be taken up by the oocyte by receptor-mediated endocytosis. In some species, at least part of the Vg is synthesized by the follicle cells, and in most species that have been examined, the follicle cells produce proteins which find their way into the egg. At the end of vitellogenic growth, the follicle cells secrete the chorion and are sloughed off as the oocyte leaves the ovariole. As already noted (Section

2.1.2), the YP of the cyclorrhaphous Diptera are different from vitellins of other insects; nevertheless, the generalities outlined above apply.

There are thus several potential control points at which JH might influence the development of the ovary: the proliferation of the oogonia/nurse cells the proliferation of the follicle cells; the previtellogenic development of the follicle; the synthesis of yolk proteins, other proteins and other macromolecules such as DNA; the entry of Vg or YP into the follicle; the uptake of Vg by the oocyte; and the production of the chorion. There have been suggestions that JH might have effects at each of these levels.

2.2.1.1 *Previtellogenic development.* Allatectomy in *Rhodnius prolixus* leads to a reduction in mitosis in the trophic primordium, a region at the apex of the ovariole in which trophocytes undergo mitosis (Pratt and Davey, 1972a). The authors interpreted these effects as indirect, due to feedback from elements lower down in the ovariole. In another reduviid, *Panstrongylus megistus*, oogonial and trophocyte mitoses are apparently under the control of factors from the brain, and are not affected by allatectomy (Furtado, 1979). In some insects, ecdysone is known to affect early events in the formation of follicles, including DNA synthesis (Hagedorn, 1983). Since it is now known that the ovaries of a number of insects, including *Rhodnius*, secrete ecdysteroids (Ruegg *et al.*, 1981), it is possible that the reduction in mitosis following allatectomy in adult females of *Rhodnius* is a consequence of the reduction in ecdysone attendant upon a very much reduced production of follicles. A similar explanation may apply in the case of a reduction in mitoses in the follicular primordium which we have noted in allatectomized females of *Rhodnius* (K. G. Davey, unpublished data). In the mosquito, *Aedes aegypti*, the increase in follicle cell number which occurs during the first 48 hours of adult life has been suggested to be due to JH (Hagedorn *et al.*, 1977), although direct evidence is lacking.

JH has been implicated in the previtellogenic growth of the follicle in several species. In *Aedes*, the formation of the follicle from the follicular primordium is under the control of ecdysone (Beckemeyer and Lea, 1980), but the subsequent growth of the follicle, which occurs in the first 3 days of adult life before the first blood meal (and hence in the absence of ecdysone), does not occur in females allatectomized at emergence, and the growth is restored by treatment with JH (Gwadz and Spielman, 1973). During this same time period, the ovary acquires competence to secrete ecdysone in response to egg development neurosecretory hormone, and this process is also under the control of JH (Shapiro and Hagedorn, 1982). A similar requirement for JH early in the process of egg development has been noted for many insects such as *Schistocerca* spp. (Strong, 1965; Tobe and Pratt, 1975), *Nauphoeta cinerea* (Lanzrein *et al.*, 1978), *Aedes atropalpus* (Kelly *et al.*, 1981), *Culex pipiens* (Spielman, 1974) and *Musca domestica* (Adams, 1974). In *Leucophaea maderae*, Koeppe *et al.* (1980a, 1980b) noted several

structural changes and an increase in DNA synthesis in the follicle cells of females decapitated at emergence and subsequently treated with JH. In *Rhodnius*, the follicle cells require exposure to JH early in development in order to acquire competence to respond to JH in the vitellogenic follicle (Abu-Hakima and Davey, 1975; see Section 3.4.2). JH also stimulates DNA synthesis in the follicle cells of *Leucophaea* (Koeppe *et al.*, 1980a; see Section 3.5.1). In summary, it is now clear that JH has an important developmental role to play in governing the differentiation of the follicle cells.

2.2.1.2 *Ovarian synthesis.* JH also governs the synthesis of proteins by various ovarian tissues. While it is generally accepted that Vg in most insects originates in the fat body, there have been reports of an ovarian source for Vg (Section 2.1.1). In *Leucophaea maderae*, JH applied *in vivo* increases the incorporation of radioactive amino acids into ovary when subsequently measured *in vitro*, and the ovary is reported to be capable of synthesizing Vg (Wyss-Huber and Lüscher, 1969, 1972). The synthesis of YPs by ovarian tissues is best known in *Drosophila*. It has been known for many years that both the fat body and the follicle cells of the ovary are capable of synthesizing YPs (Bownes and Hames, 1978; Postlethwait and Kaschnitz, 1978; Srdić *et al.*, 1979; Gutzeit, 1980; Bownes, 1982; Brennen *et al.*, 1982). YP synthesis in the ovary appears to be under the control of JH alone, and is thus different from the control in the fat body in *Drosophila*, where both JH and ecdysone stimulate Vg synthesis (Handler and Postlethwait, 1977; Postlethwait and Handler, 1979; Jowett and Postlethwait, 1980).

Proteins other than YP are also synthesized by the ovary. Several studies have identified a histidine-rich protein synthesized by the follicle cells as follicle cell product (FCP). Originally conceived as a material which enabled or stimulated the uptake of Vg by the oocyte in moths (Anderson, 1971; Anderson and Telfer, 1969, 1970), this view of its function has since been demonstrated to be unlikely (Telfer *et al.*, 1981a). Nevertheless, there are proteins which are synthesized by the follicle cells, secreted into the interfollicular spaces and taken up by the developing oocyte (Telfer *et al.*, 1981b). JH is not required for egg development in *Hyalophora*, but a similar protein occurs in *Periplaneta*, and requires JH for its synthesis (Bell and Sams, 1974). In *Leucophaea*, exposure of ovaries to JH I or JH III either *in vivo* or *in vitro* results in an increase in the general level of protein synthesis (Koeppe *et al.*, 1981b) and, while the precise site of the synthesis was not identified, it seems likely that the follicle cells were among the primary targets.

An early study (Gilbert, 1967) demonstrated that incubation of ovaries from *Leucophaea* in the presence of CA resulted in increased incorporation of palmitate into lipids, although it was not established whether the effect was on uptake of the fatty acid or on synthesis *per se*. Given that oocytes are often rich in lipids, it is surprising that these experiments have not been extended (see Section 3.2.2).

2.2.1.3 *Uptake into the follicle and oocyte.* That JH affects the uptake of yolk proteins by the ovaries has been known for many years. Early workers observed an increased formation of yolk in the oocyte near the site of the implantation of a CA or of the injection of JH (Joly, 1945; de Loof and de Wilde, 1970). In *Periplaneta americana*, injection of Vg into allatectomized females does not result in the accumulation of yolk in the ovary, while implanting CA or injecting a JH analogue restores yolk production and uptake (Bell, 1969). Working with the cockroach *Eublaberus posticus*, Bell and Barth (1971) demonstrated that previtellogenic ovaries implanted into decapitated males which were also injected with Vg would form yolk only if a CA were also implanted. In *Drosophila*, females homozygous for the *apterous*^{4} mutant synthesize YP, but fail to deposit yolk. Treatment with methoprene stimulates yolk deposition, suggesting that JH is acting upon the uptake process (Gavin and Williamson, 1976). Both methoprene and ecdysone are capable of inducing the synthesis of YPs in the isolated abdomens of female *Drosophila*, but only those abdomens receiving methoprene accumulated yolk in the oocytes (Postlethwait and Handler, 1979). Precocene-treated females of *Drosophila* exhibit decreased vitellogenesis (Landers and Happ, 1980; Wilson *et al.*, 1983), and transplanting vitellogenic oocytes to JH-deficient hosts caused the oocytes to degenerate (Wilson, 1982). These observations confirm that JH is required throughout vitellogenesis. Diapausing *Drosophila* exhibit low titres of JH, and, although YP is present in the haemolymph, it fails to enter the follicles. When diapause is terminated by appropriate environmental conditions, or by application of JH, YP enters the follicles (Saunders *et al.*, 1990). In *Locusta* (Ferenz *et al.*, 1981), JH increases the uptake of Vg into isolated ovarioles, and in *Rhodnius* (Davey and Huebner, 1974), incubation of isolated ovaries with Vg *in vitro* results in uptake of Vg only if JH is present. However, Oliveira *et al.* (1986) obtained some uptake of radiolabelled Vg into *Rhodnius* ovaries *in vitro* in the absence of JH. In *Oncopeltus fasciatus*, diapausing females accumulate Vg in the haemolymph, but do not deposit it in the ovaries. Topical application of JH analogue to starved diapausing females results in deposition of yolk in the ovaries, and a reduction of the Vg level in the haemolymph (Kelly and Davenport, 1976). Even in larvae of the gypsy moth, *Lymantria dispar*, allatectomy results in a reduction in the accumulation of extra-ovarian proteins by the ovary (Dompenciel *et al.*, 1992).

It is thus clear that JH acts directly on the ovary to influence the uptake of Vg and YP into the oocyte. It is now known that at least two processes are targets for the hormone. On the one hand, JH acts on the follicle cells, causing large spaces to appear between them (Fig. 2). This permits the Vg in the haemolymph to gain access to the oocyte surface. The development of these spaces, termed 'patency' by Pratt and Davey (1972a), is best described in *Rhodnius*, where it results from a JH-induced reduction in

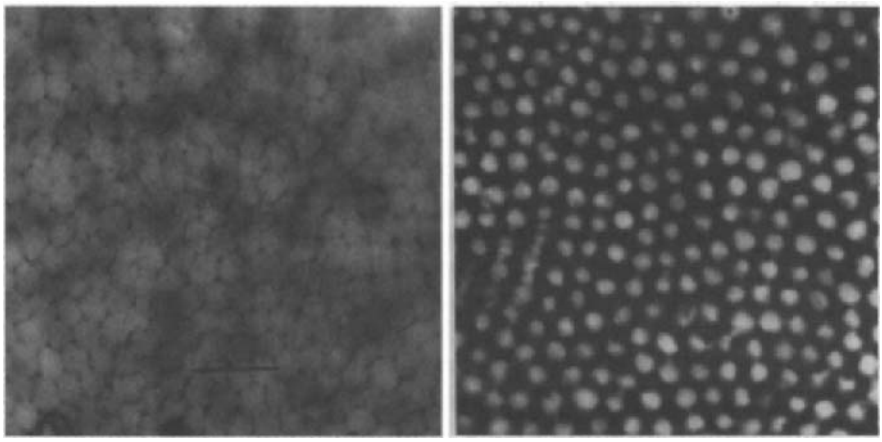

FIG. 2 Induction of patency by JH in the ovarian follicular epithelium of *Rhodnius*. Ovaries in mid-vitellogenesis were rinsed thoroughly in medium without JH over 60 min, and then transferred to medium without JH (A) or containing JH I (B) for 30 min. The connective tissue sheath surrounding the ovary was removed, and individual ovarioles were immersed briefly in the high molecular weight dye, Evans' Blue, which penetrates between the cells. In A, the spaces are very small, while in B, the follicles incubated with JH I, the spaces are large and every cell is surrounded by dye. The horizontal line in A indicates 20 μm.

volume of the follicle cells (Abu-Hakima and Davey, 1977b). This response is analysed in detail in Section 3.4.2.

On the other hand, JH also affects the receptor-mediated endocytosis at the oocyte membrane by which the oocyte sequesters the Vg. In the mosquito, *Aedes aegypti*, allatectomy at eclosion prevents the development of the endocytotic complex which normally appears in the terminal oocyte during the first 48 hours of adult life, and implantation of CA or treatment with JH III restores the development of the complexes (Raikhel and Lea, 1985). The *apterous*^{4} mutant in *Drosophila* lacks endocytotic vesicles in the oocyte membrane, and treatment with methoprene results in their appearance. Moreover, incubation of ovaries from such mutants with methoprene *in vitro* also results in development of the endocytotic complex and the formation of yolk vesicles (Tedesco *et al.*, 1981). In *Rhodnius*, exposure of membrane preparations of the oocyte to JH I increases the binding capacity of such preparations for vitellin, apparently by increasing the number of binding sites (Wang and Davey, 1992). Since this process occurs in isolated membranes, it is unlikely to be a manifestation of the same events which govern the development of the endocytotic apparatus. Perhaps the action of JH on the oocyte is another example of JH being required early in the development of a cell in order that it can undergo a more rapid specific response later.

While the secretion of the chorion is a major activity of the mature follicle cell, this process does not appear to require JH. Allatectomized females of *Rhodnius* continue to make a small number of normal eggs, albeit very slowly (Pratt and Davey, 1972a). Koeppe *et al.* (1981b) concluded, on indirect evidence, that chorion formation was unaffected by JH in *Leucophaea*. In *Aedes*, the formation of the vitelline envelope, regarded as the first step in the secretion of the chorion, will proceed in the absence of the CA, provided that the early JH-dependent development of the follicle cells has been completed, that a blood meal (administered by enema in these experiments) is available, and that ecdysone is administered (Raikhel and Lea, 1991).

A recent paper (Borovsky *et al.*, 1994a) demonstrates that in *Aedes aegypti* the ovary synthesizes JH III and other JH-related compounds in amounts which are physiologically significant. While it is uncertain whether the ovaries of other insects also synthesize JH, and while the precise identification of the compounds produced awaits confirmation, this observation has the potential to complicate the interpretation of existing experimental data. It has been an assumption of insect experimental endocrinology that allatectomy removes the source of JH, and a process which occurs in the absence of the CA has been assumed to be independent of JH. These assumptions may no longer be valid. Moreover, experiments based on the response of the ovary *in vitro* to exogenous JH are not so easily interpreted if the ovary is shown to be a source of the hormone for which it is also a target.

2.2.2 *Testes*

In many insects, all of the spermatozoa are produced before adult eclosion. In others, mature spermatozoa are present from the time of eclosion, but spermatogenesis continues during adult life. This account assumes that the process of spermatogenesis does not differ between adult and larval or pupal stages, although it is recognized that the hormonal milieu governing the process may be very different. The process of spermatogenesis proceeds in three distinct phases. There is first of all mitotic proliferation of spermatogonia for a number of divisions which is characteristic of the species. This results in a fixed number of spermatocytes in each cyst which is some power of 2. Secondly, the spermatocytes undergo meiosis. Finally, the resulting spermiocytes undergo spermiogenesis to produce the mature spermatozoa. It is also important to recognize that the testis contains a variety of tissues in addition to the germ cells. These tissues, which include the sheath which encloses the testis, the cells of the cysts which enclose the groups of developing spermatocytes, as well as a variety of 'trophic' cells such as 'apical cells' or 'basilar cells', have received rather little attention.

It has been known since the early studies of Wigglesworth (1936), who showed that mature spermatozoa continued to be produced in adult *Rhodnius* in the absence of the CA, that JH is not essential for spermatogenesis. Other

workers, while confirming that conclusion, demonstrated that allatectomy of the larval stages accelerated the rate of spermatogenesis in *Bombyx mori* (Fukuda, 1944), *Periplaneta americana* (Blaine and Dixon, 1970) and *Ephestia kuhniella* (Nowock, 1973). When additional JH was provided either by implantation of CA or by application of JH or its analogues to the penultimate instars of *Locusta migratoria* (Cantacuzène and Seureau, 1970) and *Ephestia kuhniella* (Sehnal, 1968) or to larvae of *Bombyx* (Takeuchi, 1969), spermatogenesis was inhibited. The application of juvenoids such as hydroprene to larvae of the rice moth, *Corcyra cephalonica*, resulted in a number of developmental abnormalities in the testis, but there was no evidence that this was a direct or physiological effect (Deb and Chakravorty, 1981).

A more thorough analysis conducted on *Rhodnius* larvae focused on the rate of mitosis of the proliferating spermatogonia. This analysis revealed that in cysts developing in the absence of known hormonal influence there was a low basal rate of mitosis which was stimulated by 20HE. The administration of exogenous JH had the effect of inhibiting the 20HE stimulated mitotic rate, while leaving unaffected the 'anhormonal basal level' of mitosis (Dumser and Davey, 1974, 1975a, b). While the effect of 20HE on the testis has been confirmed in *Locusta* in *in vitro* experiments (Dumser, 1980b), it is not always clear that the action of JH on mitotic rate is direct. JH is known to affect the release of the prothoracicotropic hormone (PTTH), the hormone which governs the production of ecdysone (Steel and Davey, 1985), and a high titre of JH would likely lead to a reduction in the titre of ecdysone. Nevertheless, the experiments on *Rhodnius* were conducted in the absence of PTTH. The discovery that ecdysteroids are produced by the testis of *Heliothis virescens* (Loeb *et al.*, 1984) may further complicate the interpretation of the experiments with JH.

Observations supporting a different picture from those outlined above have been made in larvae of *Papilio xuthus*, in which spermatocyte proliferation increases when active CA are implanted into insects otherwise destined to diapause (Nishiitsutsuji-Uwo, 1961). However, implantation of brains or prothoracic glands had a similar effect, so that the effect of JH may in this case be indirect via an activation of the system producing ecdysone. Incidental observations on allatectomized adult males of the orthopteran, *Nomadacris*, indicate that the surgery leads to a complete failure in spermatogenesis, but there was no demonstration that this effect was direct (Cantacuzène, 1968).

Dumser (1980a) has argued that a direct hormonal control of meiosis is unlikely in the testis of insects, although he notes that the beginning of meiosis can be delayed by autolysis of pre-meiotic stages, and that autolysis occurs in the absence of ecdysteroid. No studies have come to our attention which link JH to meiotic events.

In diapausing adults of the leafhopper, *Draeculacephala crassicornis*, application of a JH analogue, while not affecting the production of

spermatocytes, promoted the early appearance of mature sperm, suggesting an effect on spermiogenesis (Reissig and Kamm, 1975). More direct evidence for such an effect was produced by Jacob (1992), who showed that fragments of testes of the beetle, *Oryctes rhinoceros*, cultured *in vitro* required ecdysone and the testis sheath in order for mitotic and meiotic divisions to be completed. Spermiogenesis of the secondary spermatocytes produced *in vitro* would proceed only if an active CA were present in the culture medium.

2.3 ACCESSORY GLANDS

2.3.1 *Colleterial glands of female cockroaches*

Among the several types of ectadenia, or ectodermally derived diverticula of the female genital tract (Kaulenas, 1992), the colleterial glands of cockroaches have received much attention related to their regulation by JH. These glands produce the materials for the ootheca, or egg case, which results from interaction of the distinct products of the dimorphic left and right glands. The left gland produces a set of proteins (oothecins), together with calcium oxalate, protocatechuic acid-β-glucoside and other β-glucosides and a diphenol oxidase. The right gland produces a β-glucosidase. When the products of the two glands are mixed and moulded around a batch of eggs in the genital chamber, the glucosidase acts on the β-glucosides to release phenols which are then oxidized to quinones which cross-link the proteins of the nascent ootheca, causing it to harden and darken. This process is essentially similar in many species of cockroaches, although gland morphology, timing of the process and eventual degree of sclerotization of the ootheca vary with the reproductive habits of different species (Stay and Roth, 1962).

That the functioning of these accessory sex glands depends on the CA was demonstrated in *Leucophaea maderae* by Scharrer (1946). Subsequent research has been performed chiefly with the oviparous species, *Periplaneta americana*, in which it has been confirmed that the production of proteins (Bodenstein and Sprague, 1959), calcium oxalate (Stay *et al.*, 1960) and β-glucoside (Willis and Brunet, 1966) in the left gland are all under CA control. Synthetic JH I was shown to be effective in inducing protocatechuic acid β-glucoside in the colleterial gland, and this response was proposed as a quantitative assay for JH activity (Bodenstein and Shaaya, 1968); subsequent work indicated that the JH-regulated step lies in the synthesis of protocatechuic acid rather than its glycosylation, and that the primary site of this process may be in the integument (Shaaya and Sekeris, 1970). The formation of ootheca can be delayed by treating cockroaches with fluoromevalonate, an inhibitor of JH synthesis (Edwards *et al.*, 1985). Surprisingly, however, the production of the β-glucosidase in the right gland was found to be independent of the CA (Willis and Brunet, 1966).

More recently, the synthesis of the egg-case proteins (oothecins), in the left colleterial gland of *P. americana* was chosen as a suitable system for investigating the mechanism of action of JH. During the maturation of adult females, the rate of oothecin synthesis and the gland's content of total and poly(A)$^+$RNA rose steeply between days 5 and 9, while the DNA content remained unchanged (Weaver, 1981; Pau, 1981). In females allatectomized by decapitation 1 day after adult emergence, [^{3}H]leucine incorporation into oothecins, measured in glands incubated *in vitro*, was abolished, but when JH I was applied in daily doses of 2 μg synthesis was quickly restored and by day 7 had attained normal values. The *P. americana* oothecins comprise a 39 kDa protein rich in valine and proline together with a family of glycine- and tyrosine-rich proteins that fall into 5 size classes (A to E) of 14 to 28 kDa and, within classes C and D, show genetic polymorphism among individual cockroaches (Pau *et al.*, 1971; Weaver and Pau, 1987). From a cDNA library, clones coding for glycine-rich oothecins were selected for partial sequencing: the presence of repeated G doublets represented the gly codons (Pau, 1984). The deduced amino acid sequence of a 16 kDa (C-class) oothecin shows a tripartite structure, with left and right arms containing multiple repeats of the pentapeptide Gly-Tyr-Gly-Gly-Leu: features indicating homology with the chorion proteins of silkmoths (Pau *et al.*, 1986; Pau, 1987a, b). During the maturation of adult female cockroaches, the primary induction of synthesis of oothecins C and D was correlated with the rising rate of JH synthesis in the CA. The tissue content of oothecin C mRNA, assayed with the cloned probe, also rose in parallel but remained low after allatectomy on day 1 and dropped off after allatectomy on day 8. There was no correlation of oothecin synthesis or tissue mRNA with observed fluctuations in haemolymph ecdysteroid titre, and ovariectomy, which abolished a peak of haemolymph ecdysteroid, did not affect oothecin synthesis (Pau *et al.*, 1986).

In adult female *P. americana*, JH biosynthesis in the CA is cyclical, correlated with the cycles of ovarian maturation that occur at 3 day intervals (Weaver *et al.*, 1975; Weaver and Pratt, 1977). The rate of oothecin synthesis in the colleterial glands, however, once the primary induction is complete, remains relatively constant, undergoing only minor fluctuation, which is coordinate for groups C and D oothecins, during an ovarian cycle (Weaver, 1981; Pau *et al.*, 1986; Weaver and Pau, 1987). The oothecins accumulate within the gland for extrusion to the genital vestibulum at times of ootheca formation, and it is suggested that their production may be maintained by response to a relatively low level of JH and possibly by stable mRNAs (Pau *et al.*, 1987). Studies with the left colleterial gland of *P. americana* in another laboratory reported rapid fluctuations in protein synthesis and other parameters within a cycle, and an influence of ecdysterone (Iris and Sin, 1984, 1988), but it is not clear why their results differ from those of Weaver and Pau.

In *Blattella germanica*, the temporal course of events is different, since the oothecae are carried protruding from the genital pouch during a period of about 18 days between oviposition and hatching of the eggs (parturition) (Stay and Roth, 1962; Zalokar, 1968). During this interval, protein synthesis in the colleterial gland is repressed. It can be activated by removal of the ootheca, which initiates the development of a new batch of eggs (Zalokar, 1968). In females allatectomized by beheading, activation of oothecal protein synthesis by removal of the ootheca was prevented, and it could be restored by implantation of CA. Most interestingly, when synthesis of RNA and protein were assayed simultaneously during a brief incubation with [^{3}H]uridine and [^{14}C]glycine, RNA synthesis was stimulated as early as 1–2 h after removal of the ootheca, while an effect upon protein synthesis was first observed at 6 h. Incorporation of uridine into the soluble nucleotide pool was also elevated early, and Zalokar (1968) suggested that the CA hormone might stimulate nucleoside phosphorylation, but it seems that the observed effect could be a secondary consequence of activated transcription. A more recent study on *Blattella germanica* showed that after ovariectomy protein was not discharged from the left colleterial gland, but continued to accumulate up to 4 times the normal level during 42 days (Burns *et al.*, 1991). Protein accumulation was prevented by allatectomy and activated by a single injection of 0.8 μg of JH III.

The stage was set for molecular analysis of JH action in the *Periplaneta* left colleterial gland by the isolation of oothecin genes (Pau *et al.*, 1987). By probing with the cDNA clones described above, a 35 kb segment of genomic DNA was cloned which includes two complete genes for 16 kDa (C-type) oothecins. They are separated by 12 kb of DNA and transcribed in the same direction. The transcribed sequence of each extends over about 7 kb and includes two introns. 5′-Flanking DNA of one of the oothecin genes was sequenced and the intriguing observation was made that it contained two 12-nucleotide blocks having 75–100% identity with two blocks present in the upstream DNA of the JH-dependent *Vg* genes of *Locusta migratoria*, as well as similar positioning relative to the transcription start sites (Pau *et al.*, 1987). Unfortunately, the full upstream sequence of the oothecin gene has not been published, and this research has not been continued. The JH regulation of the cockroach oothecin genes clearly deserves further study.

2.3.2 *Spermathecae and associated structures*

The spermathecae are diverticula of the female genital chamber that serve to store sperm from the time of copulation until oviposition; they may secrete proteases that partially digest the spermatophore, permitting its release, as well as fluid necessary for preservation of the stored sperm (Kaulenas, 1992). In two species of grasshopper, *Chorthippus curtipennis* and *Gomphocerus rufus*, cellular differentiation and proteolytic enzyme production by the

spermatheca was reported to be prevented by allatectomy (Hartmann and Loher, 1974; Hartmann, 1978), whereas in *Locusta* and *Melanoplus* allatectomy was without effect (Kaulenas, 1992, p. 87). This difference is consistent with variation in the JH-dependence of certain other aspects of sexual function among members of the Acrididae.

In the female monarch butterfly, growth of the bursa copulatrix and receptacle gland has been shown to be stimulated by JH (Herman, 1982). Allatectomy did not affect protein synthesis in the non-homologous sperm storage organs in *Rhodnius prolixus*, however (Kuster and Davey, 1983).

2.3.3 *Male accessory glands*

There are many glandular structures associated with various parts of the male reproductive system: glands can be found associated with the testis, the seminal vesicles, and the various ducts (Gillott, 1988). This discussion is restricted to those structures of mesodermal origin which are clearly identifiable as separate glands, largely because the endocrine control of the other secretory structures has not had much attention. These accessory glands, originally important primarily in terms of the production of the spermatophore (Davey, 1960), have come to serve a variety of functions, including the production of various chemical signals to the female, such as 'fecundity enhancing' or 'receptivity inhibiting' factors (Chen, 1984). A wide variety of products, mostly, but not all, proteinaceous, have been described from male accessory glands and these may be localized in specific areas or tubular types of the glands (Davey, 1985). There are two major processes which are influenced by hormones: the development and differentiation of the glandular tissue and the control of the secretory process. While the male accessory glands in many insects are fully differentiated at adult eclosion, this is not always the case. In general, the process of differentiation is under the control of ecdysone. For a fuller discussion of the role of accessory glands, and of the control of their development and differentiation, the reader is referred to other reviews (Davey, 1985; Gillott and Gaines, 1992; Happ, 1984, 1992; Kaulenas, 1992).

Very early studies documented the importance of the CA in controlling the accumulation of secretion by the accessory glands in male insects. Thus, males of *Rhodnius* which had been decapitated posterior to the CA within 24 h of adult eclosion failed to accumulate the secretion in their accessory glands, while those decapitated anterior to the CA accumulated secretion normally (Wigglesworth, 1936). Similar complete or partial inhibition of post-eclosion accumulation of secretory products as a result of allatectomy has been noted in several species, such as *Schistocerca gregaria* (Loher, 1960), *Locusta migratoria* (Girardie and Vogel, 1966; Cantacuzène, 1967b), *Periplaneta americana* (Blaine and Dixon, 1973), *Blattella germanica* (Piulachs *et al.*, 1992), various Lepidoptera (Herman, 1975a,b; Herman and

Bennett, 1975; Herman and Dallmann, 1981), and *Aedes aegypti* (Ramalingam and Craig, 1977). Most of these studies have also documented the restoration of normal rates of accumulation of secretion by implantation of CA and/or application of JH or JH analogues.

The accumulation of the secretion in the accessory glands has been related to the JH titre in *Danaus plexippus* (Herman *et al.*, 1981) and to the synthetic capacity of the CA in *Schistocerca* (Avruch and Tobe, 1978) and *Blattella germanica* (Piulachs *et al.*, 1992).

Observations of the effect of allatectomy on the histology and ultrastructure of accessory glands confirm the more quantitative observations. The glands from various operated acridids contain little or no secretion, the epithelium is shrunken and the synthetic machinery, such as endoplasmic reticulum, free ribosomes and Golgi complexes, is comparatively underdeveloped (Cantacuzène, 1967a; Hartmann, 1971; Odhiambo, 1966a) or degenerates (De Loof and Lagasse, 1972). In *Gomphocercus*, accessory glands from last-instar larvae implanted into normal adult males develop fully and produce secretion, while those implanted into allatectomized females fail to do so (Hartmann, 1971). In *Leptinotarsa decemlineata*, the innermost layer of cells of the male accessory gland undergo autolysis after allatectomy (de Loof and Lagasse, 1972).

The requirement for JH is not always absolute. Even in the absence of the CA, there may be some accumulation of the secretory product, albeit at a reduced rate. This has been noted in particular for *Locusta* (Girardie and Vogel, 1966; Cantacuzène, 1967a, b) and for *Diploptera punctata* (Tobe *et al.*, 1979).

The principal products of the accessory glands are proteins, and some studies have shown that JH brings about an increase in protein synthesis. Allatectomy has been shown to reduce protein synthesis and JH or analogues to restore it in *Rhodnius* (Barker and Davey, 1982; Gold and Davey, 1989), *Melanoplus* (Gillott and Friedel, 1976; Venkatesh and Gillott, 1983) and *Locusta* (Braun and Wyatt, 1995). In *Periplaneta*, the number of proteins detectable by electrophoresis decreases after allatectomy. JH increases the activity of trehalase in male accessory glands of *Periplaneta* (Ogiso and Takahashi, 1984), but it is not certain that the increase in enzyme activity reflects an increase in the synthesis of this protein. In *Melanoplus*, allatectomy has quantitatively different effects on the various tubules which comprise the accessory glands (Venkatesh and Gillott, 1983), completely inhibits the production of two oviposition stimulants in the accessory secretions (Friedel and Gillott, 1976), and differentially affects the incorporation of labelled amino acids into various proteins as separated in denaturing gels (Gillott and Venkatesh, 1985). In the long hyaline gland (a tubule of the gland complex) of *Melanoplus*, a single protein, 'long hyaline protein 1' (LHP1), constitutes more than 50% of the protein content, and the synthesis of this protein is stimulated by JH III (Cheeseman and Gillott, 1988a, b).

By contrast, the composition of the proteins in the transparent accessory reproductive glands of *Rhodnius* appears not to change under the influence of JH (Barker and Davey, 1981, 1982). Thus, while it seems likely in some insects that JH may be directing the synthesis of specific proteins, there is no unequivocal evidence which demonstrates that the synthesis of any accessory gland protein is totally JH-dependent.

Only a few studies have explored the ability of JH to increase protein synthesis in male accessory glands *in vitro*. In *Drosophila*, incubation of the male accessory gland in a medium containing nanomolar JH III resulted in a nearly three-fold stimulation of protein synthesis as measured by methionine incorporation. A variety of other agents, including 20HE, failed to stimulate protein synthesis (Yamamoto *et al.*, 1988). In the transparent accessory gland of *Rhodnius*, the rate of protein synthesis as measured *in vitro* begins to decrease about the eighth day after feeding, when the secretion contained in the accessory glands has reached a maximum. Exposure of glands removed from insects at this time of decreasing levels of protein synthesis to JH I *in vitro* results in some stimulation, but glands removed from animals at times of maximum or minimum protein synthesis fail to show any increase (Gold and Davey, 1989). In both of these insects, the effect of JH on protein synthesis *in vitro* is relatively rapid, since it is detectable over an incubation period of a few hours. And in both cases, of course, the glands were taken from normal males, and thus had previous exposure to JH *in vivo*. *In vitro* studies on the male accessory glands of *Melanoplus sanguinipes* have shown that JH III can inhibit the synthesis of some proteins and induce or enhance the synthesis of others (Ismail and Gillott, 1995a). The accessory glands of males one day after emergence respond to JH by increasing the rate of synthesis of a specific protein in the long hyaline tubule, whereas effects on other proteins are not detectable until day 4. In the same system, 20HE affects different proteins.

However, in those cases in which the glands have not had previous exposure to JH, the action of JH appears to be biphasic. The best example is provided by the control of the synthesis of LHP1 in *Melanoplus*. When long hyaline glands were removed from normal males and incubated *in vitro* with JH III, the incorporation of [^{3}H]-leucine into LHP1 occurred in a dose dependent fashion over a range from 40 to 60 nM, with the response falling off at higher concentrations. When glands were removed from males which had been previously allatectomized, and incubated *in vitro* with an optimum concentration of JH III, the stimulation over a 4 h incubation was modest, and only a fraction of that exhibited by glands removed from sham-operated males. However, if the glands were pre-incubated *in vitro* overnight in JH III, the stimulation of the synthesis of LHP1 was very markedly enhanced, rising to the same level as that in similarly treated glands taken from sham-operated males. The stimulation of synthesis in glands from sham-operated males was similar in those pre-incubated or not (Gillott and Gaines, 1992).

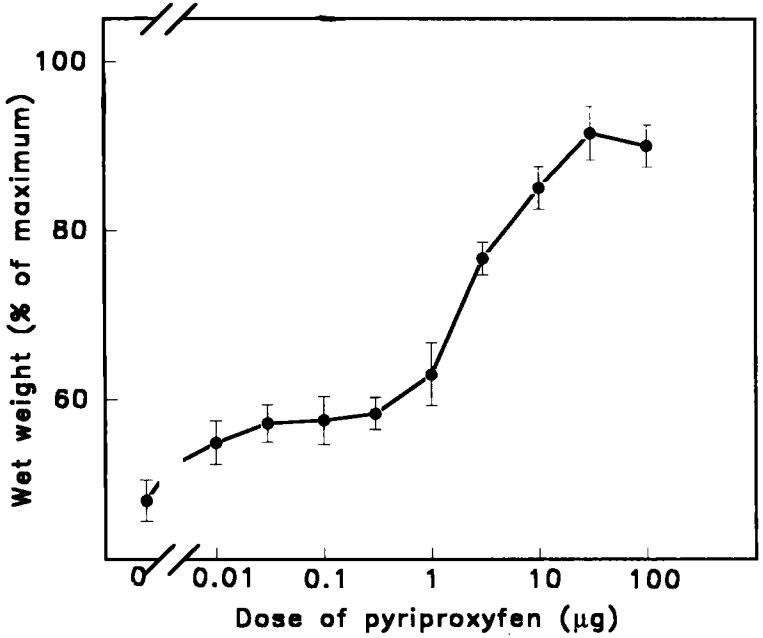

FIG. 3 Dose curve for stimulation of growth in the male accessory gland of *Locusta migratoria* by pyriproxyfen. Various doses of pyriproxyfen were applied topically in acetone to precocene-treated adult male locusts and after 72 h accessory glands were removed and weighed. Values from two experiments with different aged locusts have been normalized for different zero-dose weights. Both experiments showed a similar biphasic pattern. Values are means ± SEM (n = 6). (Data from Braun and Wyatt, 1995).

Similar observations have been made for *Locusta* (Braun and Wyatt, 1995). In males treated with precocene, the accessory glands did not grow, and further treatment with the juvenoid, pyriproxyfen, restored normal growth and stimulated protein synthesis by glands removed from treated males and incubated *in vitro*. When precocene-treated males were treated with different doses of pyriproxyfen, the growth response was biphasic (Fig. 3). A very low dose (10 ng) resulted in a small but significant increase in weight of the glands, greater doses up to 1 μg did not give any further effect, but 1–100 μg of pyriproxyfen resulted in graded increases in weight. To account for this biphasic dose response, the authors suggest that there may be two actions of JH on growth of the accessory glands.

The interpretation of all of these data related to JH is clouded to some degree by three considerations. First, the accessory glands of some insects synthesize JH. This is best known in the cecropia silkmoth where the CA of pupal males secrete the acids of JH I and II. The male accessory glands

take these up and convert them to the corresponding JHs, which are stored in large quantities in the lumen of the gland (Shirk *et al.*, 1983). While some of this material is secreted during the pupal stage, much of the hormone persists in the adult, and at least some is transferred to the female during mating (Shirk *et al.*, 1980). The content of JH in the adult male cecropia moth is exceptionally high, and these observations have been widely regarded as anomalous, but the recent report that the male accessory gland of *Aedes aegypti* synthesizes JH III and JH I as well as other JH-related compounds *de novo* (Borovsky *et al.*, 1994b) suggests that similar phenomena may be more widespread. The possibility that a tissue which responds to JH also synthesizes the hormone renders the interpretation of the effects of exogenously applied hormone difficult.

Second, there is evidence that not all of the proteins secreted by the male accessory glands are synthesized by the glands. In *Melanoplus*, the fat body and haemolymph contain proteins immunologically related to those in the accessory glands, and the glands are capable of taking up labelled proteins from the haemolymph, a process which is stimulated by JH (Friedel and Gillott, 1976). Similarly, in the stem borer, *Chilo partellus*, male accessory glands are able to take up proteins from the haemolymph *in vitro* (Ismail and Dutta-Gupta, 1990), as well as proteins from another pyralid, *Corcyra cephalonica*, but the process of uptake appears to be stimulated by ecdysterone (Ismail and Dutta-Gupta, 1990). Conversely, it is clear that in *Rhodnius* one of the proteins produced by the transparent accessory glands is secreted into the haemolymph of the male, as well as contributing to the secretions that form the spermatophore. Although this is clearly a controlled event, because the protein appears in the haemolymph well after it has been synthesized in appreciable quantities by the gland, the nature of this control, and its relation to the control of synthesis, are unclear (Sevala and Davey, 1991).

Third, there is evidence that hormones other than JH may also affect the synthesis of the secretory proteins by the accessory glands. In *Rhodnius*, ablation of the neurosecretory cells in the pars intercerebralis prevents the accumulation of protein by the transparent accessory glands, and this defect is only partly compensated for by treatment with JH I (Barker and Davey, 1981). It has been possible partially to purify a peptide from the brain of *Rhodnius* which stimulates protein synthesis by isolated transparent accessory glands *in vitro* (Barker and Davey, 1983). While these results demonstrate clearly that both JH and a neuropeptide affect the same general process, the relationship between the two apparent control mechanisms remains uncertain. A similar dual control may also exist in *Melanoplus*, where removal of the corpus allatum and the corpus cardiacum results in a greater reduction in the accumulation of secretion in the accessory glands than removal of the corpus allatum alone (Cheeseman and Gillott, 1988b). By contrast, in *Gomphocercus*, the effect of neurosecretory cells in the brain on the accessory glands is mediated by the CA (Hartmann, 1971; Hartmann *et al.*, 1972). As already noted, the development

of the glands is under the control of ecdysteroid, and in some species it has been suggested that 20HE controls the secretion of the mature glands (see Gillott and Gaines, 1992 for references).

Although there are many points of variation and many uncertainties, enough is known to justify the general conclusion that the accessory reproductive glands of the male constitute a target for the action of JH, which appears to act by stimulating the synthesis of some or all of the proteins that are secreted by the glands. There are parallels between the action of JH on protein synthesis in the male glands and its action on the fat body. In both cases, tissues which have not had recent exposure to JH require a longer exposure to JH before the response in terms of protein synthesis becomes manifest. In both cases, there is evidence of dual control of protein synthesis, by JH and by neuropeptides. However, there is not as yet clear evidence that any protein in the accessory glands is absolutely dependent on JH for synthesis: JH affects the rate of synthesis of some proteins, but in the absence of JH, small quantities of these proteins are nevertheless produced. As will be seen in Section 3.4.1, our knowledge of the molecular actions of JH on the accessory glands is even more rudimentary than our knowledge of its actions in controlling the synthesis of Vg in the fat body.

2.4 FLIGHT MUSCLE

The flight muscles constitute a major tissue of adult pterygote insects, the development and maintenance of which require a substantial commitment of resources. Growth and functional maturation of the flight muscles usually continues for several days or a week after eclosion, and this appears to be a developmentally programmed process, not linked to reproductive maturation, and not under direct hormonal regulation (Finlayson, 1975). In some insects, the flight muscles subsequently degenerate when no longer needed, liberating nutrients which can contribute to reproduction. This degeneration may be complete and permanent, as in termites or queen bees after the nuptial flight, but in insects of several kinds the flight muscles undergo cyclic degeneration and regeneration correlated with reproductive activity and/or diapause. Evidence from several species shows that JH has an intimate role in both permanent and reversible flight muscle degeneration. The topic has been reviewed by Finlayson (1975), Pener (1985) and Rankin (1989).

2.4.1 *Crickets*

Young adults of the house cricket, *Acheta domesticus*, after a few days of flight activity, adopt a terrestrial life style, the flight muscles break down and they commence reproduction. Following allatectomy in the last larval instar, the flight muscle degeneration, which is normally complete by day 8 of adult life, was prevented, and no loss of muscle volume had occurred in senescent

50-day old crickets (Chudakova and Gutmann, 1978). Histochemical enzyme assays showed that the normal loss of ATPase and succinate dehydrogenase in degenerating muscles was prevented by allatectomy, as well as an increase in acid phosphatase which could be detected in normal crickets one day earlier than histologically recognizable muscle fibre degeneration.

In the wing-dimorphic cricket, *Modicogryllus confirmatus*, either natural shedding or experimental removal of the hindwings is followed by flight muscle histolysis and increased egg production. Application of methoprene to either intact or neck-ligated long-winged adults caused initiation of muscle degeneration and egg production, whereas administration of JH III stimulated muscle histolysis without any visible effect on ovarian development (Tanaka, 1994). This is interpreted as indicating that flight muscle breakdown can be triggered by brief exposure to JH or an analogue, whereas ovarian development requires a longer exposure which was not provided by a single dose of the biologically labile JH.

2.4.2 *Cotton stainers* (Dysdercus *spp.*)

In several species of the plant-feeding bugs, *Dysdercus* (Pyrrhocoridae), the choice of reproduction or dispersal by flight is determined largely by the availability of food. Feeding of *D. fulvoniger* on cotton seed initiates a reproductive phase, which includes flight muscle histolysis and vitellogenesis in females; in males, flight muscle histolysis is restricted to a few old individuals (Davis, 1975). Antigens from degenerating flight muscles have been detected in developing oocytes (Nair and Prabhu, 1985b). Both flight muscle histolysis and vitellogenesis were prevented after allatectomy, in spite of feeding, and implantation of CA from reproducing females into unfed virgin females brought on both processes. Topical application of JH I, without feeding, was effective in bringing about muscle histolysis and egg maturation in females, as well as muscle histolysis in some older males. Denervation of the flight muscles also led to histolysis without feeding. While it is clear that JH is responsible for the initiation of flight muscle histolysis in *D. fulvoniger*, it is possible that its action may be mediated by the nervous system (Davis, 1975). In another species, *D. cingulatus*, experiments with allatectomy, CA implantation, the JH analogue kinoprene (ZR777) and precocene indicate a similar picture, with both vitellogenesis and flight muscle degeneration being dependent on JH, but they shed no further light on the question whether JH acts directly on the muscle (Nair and Prabhu, 1985a).

2.4.3 *The pea aphid*

In the pea aphid, *Acyrthosiphon pisum*, the flight muscles of alate adults normally break down when larviposition commences after migratory flight and feeding (Kobayashi and Ishikawa, 1994). When starved aphids were treated

with pyriproxyfen the protein content of their flight muscles decreased and they lost the ability to fly, and application of precocene was effective in preventing normal muscle breakdown. It was found that both the normal and JH analogue-induced muscle degeneration were accompanied by accumulation of ubiquitin-conjugated proteins, suggesting that ubiquitin-dependent proteolysis is an aspect of flight muscle cell death.

2.4.4 *Bark beetles*

Whereas the degeneration of the flight muscles in the insects just described is permanent, following the end of a flight phase in the life cycle, other instances exist where flight muscle involution is fully reversible, in coordination with reproductive cycles. In the bark beetles and ambrosia beetles (Scolytidae), after dispersal by flight, the adults bore into logs, mate, oviposit and then re-emerge for additional cycles of flight and reproduction. In *Ips paraconfusus* (= *confusus*), within a few days after attack on a log the flight muscle volume in both females and males is reduced by about 75%, and it remains low during the tunnelling phase, to be restored after re-emergence. Muscle volume reduction could be induced within 2 days by application of JH III (Borden and Slater, 1968), and topical application of a JH analogue (ZR-615) to young adult beetles also induced rapid reduction of the flight muscles (Unnithan and Nair, 1977). Cytological changes were the same in naturally and experimentally atrophying muscle: myofibrils were reduced in size and mitochondria showed degenerative changes, but the nuclei, sarcolemma, tracheoles and nerve endings remained intact in preparation for regeneration. The process is anatomically specific, as leg muscles show no parallel changes.

In the Douglas fir bark beetle, *Dendroctonus pseudotsugae*, application of a JH analogue was followed by an increase in histochemically demonstrated acid phosphatase sites in the indirect flight muscles, and when muscle was incubated *in vitro* with the JH analogue changes were reported within 6 h that did not appear in the controls (Sahota, 1975). The increase of acid phosphatase activity *in vivo* was blocked by injection of eserine, and the results suggest some interaction of neural stimuli with a direct effect of JH in bringing on flight muscle degeneration. In the spruce bark beetle (*D. rufipennis*), the muscle degenerative changes that normally follow entry into logs were prevented by application of precocene, which presumably blocked JH production (Sahota and Farris, 1980).

2.4.5 *Colorado potato beetle*

The Colorado potato beetle, *Leptinotarsa decemlineata* (Chrysomelidae), has a reproductive diapause resulting from CA inactivity brought on by short day length, whereas JH production resulting in reproductive activity is induced by long day conditions (Section 2.1.1.6). At the onset of diapause, the beetles burrow into the ground and the flight muscles degenerate: the myofibrils

become greatly reduced in diameter and most of the mitochondria ('sarcosomes') break down (Stegwee *et al.*, 1963). At the end of diapause, when the beetles emerge from the soil, these processes are reversed, with the myofibrils becoming thicker and the mitochondria increasing greatly in number and size. The extreme morphological and biochemical changes undergone by the flight muscle mitochondria have been studied (Stegwee, 1964). The degenerative and regenerative changes were reproduced by allatectomy and CA implantation, respectively. The process is thus essentially similar to that observed in scolytid beetles with the important difference that the role of JH is reversed: in bark beetles muscle degeneration is induced by JH, whereas in *Leptinotarsa* degeneration follows withdrawal of JH. This relationship is fitting for the respective life styles of the two types of beetles, but the mechanism by which JH exerts opposite effects requires explanation (Section 3.5.3).

2.5 THE NERVOUS SYSTEM: BEHAVIOUR

For many years, evidence has been accumulating that the CA and their hormonal products exercise diverse effects on the behaviour – chiefly related directly or indirectly to reproduction – of adult insects (reviews: Barth and Lester, 1973; Truman and Riddiford, 1974). We shall briefly review the known behavioural effects of JH in different groups of insects, and then consider some recent studies which are moving to the level of cellular effects in the insect nervous system.

2.5.1 *Female sexual behaviour*

The sexual behaviour of female insects typically comprises three phases (Truman and Riddiford, 1974; Barton Browne, 1993):

1. primary defensiveness, in which the virgin female is not yet ready to mate, does not send out signals to attract males, and repels any approaches made by males;
2. receptivity, usually involving calling (chemical, visual or auditory), leading to copulation, and
3. secondary defensiveness, in which mating is again rejected, either permanently or for a limited period (for example, until oviposition has taken place).

The neuroendocrine system plays the essential role of responding to stimuli such as day-length, temperature or intake of food, and inducing appropriate, coordinated responses both in reproductive behaviour and in the physiology of the gonads and accessory glands. While a variety of neuropeptides may be involved in signal transduction, the chief factor in bringing about the transition from state (1) (primary defensiveness) to state (2) (receptivity) is often JH. The

role of JH is that of a modifier (Truman and Riddiford, 1974) that induces the receptive state, and the actual behaviour is then released by some immediate stimulus. This 'modifying' action of JH falls within the broader category of preparation of tissues for subsequent regulatory responses, that we describe, later in this review, as 'priming' (Section 3.3).

The degree to which female reproductive behaviour depends upon JH varies widely in different insects. In those species in which oogenesis is completed in the pharate adult, and the adult is short-lived and ready to mate soon after eclosion, it is advantageous to mate as soon as possible and hormonal control is unnecessary (Barth, 1965). Sexual receptivity in insects, including the role of JH, has been reviewed recently (Ringo, 1996).

2.5.1.1 *Orthoptera.* Early experiments on different species of grasshoppers and locusts, reviewed by Barth and Lester (1973) and Truman and Riddiford (1974), illustrate how greatly the dependence of female sexual behaviour on the CA can differ within a single family. Among Acrididae, in *Locusta migratoria*, *Nomadacris septemfasciata* and *Chortohippus curtipennis*, allatectomy did not diminish female receptivity to mating, whereas in *Syrbula fuscovittata*, *Gomphocercus rufus* and *Schistocerca gregaria* (Strong and Amerasinghe, 1977) receptivity was abolished and the allatectomized females remained permanently defensive. In *G. rufus*, sexual receptivity was restored by injection of JH (Hartmann, 1978). Recent work on the same species, however, has shown that sexual behaviour patterns are correlated with rates of JH III production by the CA, and not with haemolymph JH levels, and the data indicate feedbacks between behaviour patterns and CA activity (Hartmann *et al.*, 1994).

Among crickets of several species, female sexual receptivity was not abolished by allatectomy, although response times and thresholds were often increased (Renucci *et al.*, 1985; Loher *et al.*, 1992). In *Gryllus bimaculatus*, however, egg production and oviposition were greatly reduced by allatectomy and were restored by treatment with methoprene (Orshan and Pener, 1991).

Phonotactic response of female crickets: The influence of the CA upon the phonotactic response of females to the male calling song in *Acheta domesticus* (Stout *et al.*, 1976) has provided the basis for intensive study that has led to the identification of neurons that are apparently directly affected by JH. The directionality of phonotaxis of mature females in response to electronically simulated male calling song, as well as their readiness to copulate, deteriorated after allatectomy and were enhanced by JH III or methoprene, significant effects being observed as early as 6 h after topical administration (Koudele *et al.*, 1987). In further analysis (Walikonis *et al.*, 1991), the threshold for positive phonotaxis to simulated calling song was found to decrease from over 95 dB in day 0 females to 55 dB on day 3, and this change was correlated with increasing JH synthesis in the CA. The range of syllable periods to which

females responded also increased with age, so that older females became less selective, and syllable period selectivity could also be restored by administration of JH III.

The phonotactic response threshold was shown to depend on prothoracic ganglion auditory interneuron L1, which must encode the temporal structure of the male's calling song. Unilateral inactivation of L1 resulted in crickets moving circularly, as if calling song were perceived with only one ear (Atkins *et al.*, 1992). During days 2 to 4, the phonotactic threshold of adult female crickets and the response threshold of L1 (measured by intracellular recording) decreased in synchrony; application of JH III to day 1 females caused the L1 threshold to drop precociously (within 12 h) to the level normally found on day 3. This effect of JH was blocked by the transcriptional inhibitor, α-amanitin, or the translational inhibitor, emetine (Stout *et al.*, 1991). Most impressive is the fact that when JH III was applied directly to the prothoracic ganglion a sharp lowering of the phonotactic threshold was observed within 2 h, whereas similar treatment of the metathoracic ganglion was without effect (Fig. 4). Application of JH to the right or left half only of the prothoracic ganglion resulted in the cricket's circling in response to the calling stimulus (Stout *et al.*, 1991). The authors conclude that the evidence indicates a hormonally mediated genetic regulation of the responsiveness of neuron L1.

Further studies were related to selectivity for syllable period, which is the most important temporal parameter of calling song in *A. domesticus*. Here, prothoracic interneuron L3 has been found to be involved (Henley *et al.*, 1992). In young adult females, L3's response to successive syllables in a chirp decreases by as much as 50%, and the degree of decrement is greatest in response to calling songs having favoured syllable periods, so that the decrement in L3's response is an indicator of syllable period selectivity. It was found that the L3 decrement diminished with age of the cricket, but in old individuals it could be restored by application of JH III. JH is therefore responsible for the syllable period selectivity of neuron L3. In contrast to the rapid effect (2 h) of JH III observed for L1, however, the effect on L3 was observed only 2–4 days after application of the hormone.

In spite of the different hormone response kinetics for L1 and L3, it has been suggested that the activity modulation of both neurons by JH may result from induction of membrane neurotransmitter receptor proteins, possibly for acetylcholine (Henley *et al.*, 1992; Atkins and Stout, 1994). A DNA probe derived from the sequence of the α-L1 nicotinic ACh receptor gene of *Schistocerca gregaria* was used to assay for expression of the corresponding gene in the *Acheta* prothoracic interneurons by *in situ* hybridization (Stout *et al.*, 1992, 1993). In the soma of L1, the receptor mRNA was more abundant in older females with low acoustic thresholds than on day 1; it was reduced after allatectomy and increased after treatment with JH III. Thus, the observations were consistent with the hypothesis that the regulation of phonotactic sensitivity depends on JH stimulated expression of nicotinic receptors in the L1

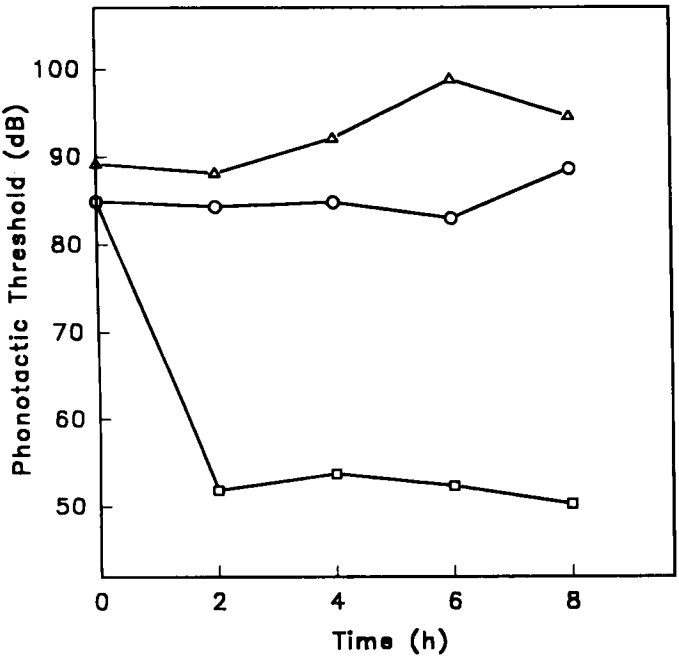

FIG. 4 Lowering of phonotactic threshold in *Acheta domesticus* by JH III applied to the prothoracic ganglion. In 1-day-old adult female crickets, 5 μg of JH III in 1 μl olive oil, or 1 μl olive oil vector, was applied directly to exposed thoracic ganglia for 3 min, then washed off with pure olive oil. Phonotactic thresholds (directed walking in response to a model calling song of varied intensity) were measured at 2 h intervals, □ Prothoracic ganglion, JH; △, prothoracic ganglion, oil control; ○, metathoracic ganglion, JH (unresponsive). (Data from Stout *et al.*, 1991).

neuron. In the L3 neuron, receptor mRNA content was similarly correlated with the natural or applied levels of JH III. JH III can therefore increase the excitatory input into both neurons, and it remains to be determined how this effect is related to their different roles in the discrimination of calling song. An additional observation of potential importance is the presence of a 23 kDa JH binding protein in the *Acheta* prothoracic ganglion, that is not found in the metathoracic ganglion (Stout *et al.*, 1992).

Oviposition behaviour in Acheta: Studies on the oviposition behaviour of *Acheta domesticus* have led to the identification of a different kind of action of JH on the nervous system. Virgin adult female crickets engage in movements of the ovipositor resembling oviposition even though no eggs are laid. Through continuous recording, it was determined that these ovipositor movements are totally abolished in adults that had been allatectomized before the imaginal moult, but after injection of JH III movements ensued with intensity equal to those in intact females (Renucci *et al.*, 1988, 1992). Since there was a variable

interval of several days between JH injection and the first ovipositor movements, the effect of the hormone is described as a primer effect. The possible nature of this effect has been found in recent work which showed that mitosis of neuroblasts in the mushroom bodies of the adult cricket brain is stimulated by JH (Cayre *et al.*, 1994). These bodies are a major integrative centre of the insect brain, and the authors suggest that the JH-mediated cellular changes could provide the basis for hormonal control of oviposition behaviour.

2.5.1.2 *Dictyoptera.* In cockroaches, courtship and mating depend on contact pheromones from the integument of the female, and the production of these has been shown in several species to depend on the CA. Mating behaviour itself, however, is generally independent of JH. Thus, in *Byrsotria fumigata*, allatectomized females did not elicit courtship by males unless they were coated artificially with the sex pheromone; after this treatment allatectomized females mated normally (Roth and Barth, 1964). In several species of cockroach, although female receptivity is correlated with the cycle of CA activity, tests after allatectomy showed that JH is not required. This has been observed with *Nauphoeta cinerea*. In *Leucophaea maderae*, mating was significantly delayed after allatectomy, although in tests in another laboratory this effect was not observed, possibly reflecting strain differences (Engelmann and Barth, 1968). Recent experiments with *Blattella germanica* and *Supella longipalpa* support the hypothesis that JH controls female receptivity in these species (Schal and Chiang, 1995).

Dependence of sex pheromone output on JH has been demonstrated by the failure of females to attract males after allatectomy, and restoration of attractiveness after implantation of CA or treatment with JH or analogue, in *B. fumigata*, *Blaberus discoidalis*, *Pycnoscelus indicus* and *Periplaneta americana* (review: Barth and Lester, 1973). More recently, a study on the oviparous cockroach, *Supella longipalpa*, has differentiated between pheromone production, measured by assaying the male response to extracts from female insects, and calling, observed as elevation of the wings and abdomen, and attractiveness to males (Smith and Schal, 1990). Both processes were prevented by allatectomy and restored by implantation of CA or application of hydroprene to the paper lining the dishes in which the insects were kept. In intact females, the mean age of initial pheromone production was advanced by application of low doses of hydroprene, but very high doses inhibited both pheromone production and calling.

Analysis of the role of JH in pheromone output has been carried further using *Blattella germanica*, a species in which the ootheca is carried by the female for 22 days until the eggs hatch, during which interval JH production in the CA is inhibited (Schal *et al.*, 1990). *B. germanica* secretes a non-volatile contact pheromone made up of three C-31 ketones, of which the major component, 3,11-dimethyl-2-nonacosanone, was quantified in cuticular ex-

tracts by gas chromatography. Production was unaffected by mating or ovariectomy, but was drastically lowered after decapitation, head-ligation, allatectomy or treatment with high levels of precocene, and after these operations it could be restored by application of hydroprene. The biochemical pathway of pheromone synthesis has been studied and rates of synthesis measured by the incorporation of [^{14}C]propionate (Schal *et al.*, 1991). During a gonotrophic cycle, the rate of pheromone synthesis was closely correlated with the rate of JH release by incubated CA, and in JH-deprived animals it was related to the dose of administered hydroprene. The stimulation of pheromone synthesis by the JH analogue was smaller in starved or headless insects than after allatectomy, from which an effect of feeding, in addition to the effect of JH, on pheromone synthesis is proposed (Schal *et al.*, 1991).

In addition to the non-volative contact pheromone just described, *B. germanica* females have recently been shown to produce a volatile sex pheromone that is released in a characteristic calling behaviour (Liang and Schal, 1993a, b). Both the synthesis of the volatile pheromone and the calling behaviour are dependent on JH (Liang and Schal, 1994). Calling was abolished by allatectomy but reinstated by application of fenoxycarb. Although a threshold titre of JH is required, however, it is not the sole requirement for calling, since calling is immediately terminated by mating and cannot be initiated by administration of a JH analogue during the 3-week period when an ootheca is being carried. A dual control of calling, with a permissive role of JH, subject to inhibitory neural signals, is proposed (Liang and Schal, 1994).

2.5.1.3 *Lepidoptera.* In many species of moth, oogenesis is largely completed by the time of adult eclosion, the adults do not feed, and calling and mating can occur in the first day of adult life: in these cases, control by JH is not expected. This is borne out by evidence for several saturniid silkmoths and the waxmoth, *Galleria mellonella* (review: Barth and Lester, 1973), as well as *Manduca sexta* (Sasaki and Riddiford, 1984; Itagaki and Conner, 1986) and the gypsy moth, *Lymantria dispar* (Hollander and Yin, 1985; Tang *et al.*, 1987). In these species, pheromone release and copulation proceed normally despite allatectomy in the pupal stage.

Some other Lepidoptera have longer-lived, feeding adults in which reproductive maturation and activity are regulated in response to environmental and physiological signals. This is the case with many members of the Noctuidae, and an effect of precocene in reducing sex attractancy and mating, implying involvement of JH, was first observed in both sexes of the armyworm, *Mythimna (Leucania) separata* (Fu *et al.*, 1986). In the true armyworm, *Pseudaletia unipuncta*, low temperature and short day-length are associated with migration and delayed reproductive activity, and this effect has been shown to be mediated by JH (Cusson and McNeil, 1989). Another migratory noctuid, the black cutworm, *Agrotis ipsilon*, also uses JH regulation, since calling and mating, as well as ovarian maturation, are prevented by

decapitation or allatectomy, and stimulated after these operations by treatment with fenoxycarb (Gadenne, 1993); the effect of JH in this system appears to be upon release of a pheromone biosynthesis activating neurohormone (PBAN) from the brain (Picimbon *et al.*, 1995). Analysis of activities in *P. unipuncta* (Cusson *et al.*, 1994a) indicates that the effect of JH on pheromone production is complex: JH above a certain threshold is apparently a prerequisite for the release of PBAN from the brain-CA and for calling behaviour, and these events are subject to circadian timing by a neural diel rhythm; JH is also responsible for the development of biosynthetic activity in the pheromone gland. The role of JH in both the neural and the glandular tissue is therefore in the nature of priming.

In the omnivorous leafroller (*Platynota sultana*), a different effect of JH has been described (Webster and Cardé, 1984). Mating normally provides the signals for cessation of calling and onset of oviposition, but in intact virgin females these behavioural changes can be precipitated by treatment with JH or hydroprene. The authors suggest that JH transferred from the male in copulation (as is known to occur in *Hyalophora cecropia*) may provide this signal, but direct evidence for this is required.

2.5.1.4 *Coleoptera.* In three species from this important order, the bark beetle, *Ips confusus* and *I. duplicatus* (Borden *et al.*, 1969; Ivarsson and Birgersson, 1995), and the mealworm beetle, *Tenebrio molitor* (Menon, 1970), female sex pheromone production has been shown to be under the control of the CA and inducible by JH or analogues. In the burying beetle, *Nicrophorus orbicollis*, ovarian maturation is delayed until carrion suitable for burying is encountered. The discovery of carrion releases specific behaviours, accompanied by a rapid surge in JH titre in the haemolymph, which more than doubles within 10 min (Trumbo *et al.*, 1995), and the authors suggest that the behaviours depend on the elevated JH titre.

2.5.1.5 *Diptera.* In the few dipterans that have been tested, female receptivity was found to depend on JH. In *Drosophila melanogaster*, injection of CA into female pharate adults induced early mating, suggesting an effect of JH on sexual receptivity (Manning, 1966), and this conclusion is supported by the observation that females of the JH-deficient *apterous* mutant exhibit reduced receptivity (Ringo *et al.*, 1991). Cuticular long-chain hydrocarbons as contact pheromones play a major role in attractivity of *D. melanogaster*, and JH is found to influence synthesis within this group, although another cephalic factor exerts the main control over pheromone production (Wicker and Jallon, 1995). In the mosquito, *Aedes aegypti*, and the house fly, *Musca domestica*, mating activity was reduced by allatectomy and restored by application of JH or an analogue (review: Barth and Lester, 1973), and in the blowflies, *Lucilia cuprina* and *Calliphora vomitoria*, application of methoprene greatly increased female sexual receptivity (Barton Browne *et al.*, 1976; Trabalon and Campan, 1985).

2.5.2 *Male sexual behaviour*

The sexual behaviour of male insects is much less often dependent upon the action of JH than that of females, although again there is wide variation among species (reviews: Barth and Lester, 1973; Truman and Riddiford, 1974). Among the Acrididae, the family most frequently studied, male sexual activity is regulated by JH in species that have a reproductive diapause determined by photoperiod: thus, copulation is inhibited after allatectomy in *Schistocerca gregaria*, *Nomadacris semptemfasciata* and *Oedipoda miniata*, which have reproductive diapause, but not in a group of non-diapausing grasshoppers. It was inhibited completely in the diapausing *Locusta migratoria cinerascens* but only partially, perhaps as a consequence of generally reduced activity, in the non-diapausing *L. m. migratorioides*. Recent thorough experiments with the crickets, *Gryllus campestris* and *G. bimaculatus*, failed to obtain any evidence for participation of JH in male agonistic and sexual behaviour (Adamo *et al.*, 1994). In several species of cockroaches, the CA are not required for male sexual activity, although in *Supella supellectilium* it is reported that male sexual behaviour and aggressiveness were abolished after allatectomy and transiently restored after CA implantation, possibly through effects on pheromone production (Pathak and Mukerji, 1989; Schal and Chiang, 1995).

Recently, certain noctuid moths that have reproductive and migratory phases determined by photoperiod and temperature have been examined, since hormonal regulation seemed likely. In males of the black cutworm, *Agrotis ipsilon*, responsiveness to the female sex pheromone was induced after treatment with fenoxycarb and prevented after allatectomy; since antennogram recordings were not affected by allatectomy it is concluded that JH must act at the level of the central nervous system (Gadenne *et al.*, 1993). Similarly, in the true armyworm, *Pseudaletia unipuncta*, male receptivity shows a correlation with rates of JH biosynthesis which suggests dependence on JH action (McNeil *et al.*, 1994).

In the bark beetle, *Ips confusus*, however, the male is the producer of sex pheromone, and application of JH III to male beetles was shown to induce sex pheromone production in the hindgut and attractiveness to females (Borden *et al.*, 1969). The boll weevil, *Anthonomus grandis*, produces sex pheromones in the male fat body, and production of identified pheromones by fat body *in vitro* was increased by the presence of JH III, especially when the plant substance, β-bisabolol, was also present (Wiygul *et al.*, 1990).

2.5.3 *Division of labour in social bees and wasps*

Among honey bees and some other eusocial insects, the workers exhibit age polyethism, or the successive dedication to different tasks with increasing age. Honey bee workers up to about 3 weeks old serve chiefly as nurses and perform other duties within the hive, and thereafter they embark on duties outside the

hive, including foraging. The physiological and genetic basis of this transition has been intensively studied and it has been found to be regulated by JH, which apparently modulates behavioural response thresholds (reviews: Page and Robinson, 1991; Robinson, 1992). In the adult worker bee, the haemolymph titre of JH III rises with age, owing to increasing rate of synthesis by the CA, and the early low titres are associated with the performance of hive duties, whereas the higher titres that are found later are associated with field duties. Treatment with JH or analogues brings on precocious foraging. High JH titres repress Vg synthesis (Section 2.1.1.7) and also cause degeneration of the hypopharyngeal glands, which secrete larval food and normally degenerate at the time of the switch to outside duties. In colonies having experimentally modified age structure, workers could adapt to perform duties that were not typical of their temporal age, and these individuals were found to have JH titres characteristic of their behavioural role, not their chronological age, suggesting that altered JH synthesis can be the mediator of an environmental signal for modified behaviour (Robinson *et al.*, 1989). The shift from foraging behaviour is accompanied by an increase in volume of the neuropil in the mushroom bodies of the brain; this neural development could be brought about in young bees by treatment with methoprene, but was also affected by flight experience (Withers *et al.*, 1995). Despite the accumulated evidence indicating that JH is a mediator of age polyethism, this theory has recently been questioned as a result of the failure to bring about alteration of wax production by various treatments designed to alter the JH level in bees (Muller and Hepburn, 1994).

A specific behaviour that is modulated by JH is the response to alarm pheromone, the threshold for which is raised by JH analogue treatment (Robinson, 1987). Since sensory perception, assayed by electroantennograms, was unaltered, the effect of JH appears to reside in central nervous system response thresholds.

Among bees and wasps, JH-mediated age polyethism appears to be characteristic of advanced eusociality, rather than a particular taxonomic group. In the highly eusocial wasp, *Polybia occidentalis*, application of methoprene to young adult workers induced precocious out-of-nest behaviour (O'Donnell and Jeanne, 1993), whereas in the primitively eusocial bumble bee (*Bombus terrestris*) treatment with JH or methoprene did not affect worker nest or foraging activities (Cameron and Robinson, 1990).

2.5.4 *Flight and migration*

Another aspect of the behaviour of adult insects that is strongly influenced by JH is flight activity, especially in migratory species (reviews: Pener, 1985; Rankin *et al.*, 1986; Rankin, 1991). Early experiments with locusts showed that phototactic activity in adult *L. migratoria* (Cassier, 1963, 1964) and general locomotor activity in *S. gregaria* (Odhiambo, 1966b) were stimulated by the

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is dependent on JH (Lessman *et al.*, 1982). JH titres are high in summer and low during the winter reproductive diapause, and the spring and fall migrations are associated with changing JH titres (Lessman and Herman, 1983). The response to JH appears to be altered by effects of environmental factors, and in late winter butterflies, after allatectomy, flight activity can be stimulated by JH therapy (Rankin *et al.*, 1986; Rankin, 1991). In another migratory lepidopteran, the armyworm, *Pseudaletia unipuncta*, migration is associated with lower JH production than is reproductive activity, and the existence of different roles for the several JH homologues and JH acids that are produced by the CA is a possibility under consideration (McNeil *et al.*, 1994). Flight activity is also influenced by other hormones: adipokinetic hormone (AKH; in energy supply) and octopamine (in enhancing neuronal activity) (Rankin *et al.*, 1986). In summary, while it is clear that JH is an important component in establishing migratory flight, it is also clear that other environmental, physiological and endocrine influences are involved, and the precise interactions that determine migration are not yet understood for any species.

2.5.5 *Feeding behaviour*

Newly emerged adult mosquitoes, *Culex pipiens*, require a nectar meal before they become responsive to an animal host. Recent research shows that the switch from preference for honey odour to bird odour could be brought about, without sugar feeding, in a dose-dependent manner by application of methoprene (Hancock and Foster, 1992). Since previous work had shown that nutrition was important for the post-emergence rise in JH titre, it is inferred that JH mediates the acquisition of host-seeking behaviour.

2.5.6 *Feedback on neurosecretory cells*

JH exercises feedback effects on its own biosynthesis which imply actions in the nervous system. The production of JH in the CA is regulated by both positive and negative signals in the form of neuropeptides (allatotropins and allatostatins, respectively) released from neurosecretory cells in the brain (Tobe and Stay, 1985). Among the signals that are integrated by the brain for appropriate regulation of allatotropin and allatostatin output is the haemolymph level of JH itself. Assays of rates of JH output by CA after administration of JH analogues show that both stimulation (from low doses of JHA) and inhibition (from high doses) can be obtained, but JH does not appear to act directly on the CA. Recent experiments, in which allatostatin output in *Diploptera punctata* was measured by radioimmunoassay, show that a JH analogue acts on the brain to stimulate release of allatostatins (Stay *et al.*, 1994). Although it is likely that JH acts on the neurosecretory cells themselves, this has not yet been proven.

3 Cellular and molecular actions of juvenile hormone

In the preceding sections, JH is revealed as coordinating a wide variety of activities involving many different tissues in the adult insect. As already noted, the analysis of cellular and molecular mechanisms by which JH regulates the activities of its various targets has thus far not kept pace with the description of the biological effects. In this section, we shall examine the progress that has been made in this direction, using principally three insect tissues, the ovarian follicular epithelium, the male accessory gland, and the fat body, together with a more limited reference to the thoracic flight muscles.

3.1 JH BINDING PROTEINS AND PUTATIVE INTRACELLULAR RECEPTORS

3.1.1 *Objectives and experimental approaches*

The specificity and sensitivity of hormone action require that a hormone act through binding to a specific, high-affinity site on a receptor, from which the signal is somehow transduced into specific modification of cellular activity. Among biological macromolecules, only proteins possess the conformational specificity and flexibility needed for this function. To understand how a hormone works, therefore, identification and characterization of its protein receptor(s) is crucial, but for JH this goal has been remarkably elusive. Among the hazards that beset the path are the propensity of JH and analogues for non-specific binding, and the ubiquity of JH-binding proteins (JHBPs) having a transport role and JH-metabolizing enzymes, both of which have high-affinity, specific JH-binding sites and can occur at high abundance, relative to that expected for a hormone receptor. The evidence is strongly suggestive that JH can act, in certain insect tissues, both at the cell membrane (Section 3.4) and within the nucleus (Section 3.5), and two types of JH receptor appropriately located in the cell are therefore to be expected. By inference from the knowledge of hormone receptors in vertebrate systems, these are likely to represent distinct families of proteins. The current state of research on membrane receptors for JH will be discussed in Section 3.4; here, we shall first summarize what is known about haemolymph JH carrier proteins and then address the status of efforts to identify intracellular receptors. Fuller information, including underlying principles and the historical development of this field, is available in reviews (Goodman and Chang, 1985; Koeppe and Kovalick, 1986; Goodman, 1990; Trowell, 1992).

As in other areas of biomolecular research, the results obtained are highly dependent on the methods used. The classical approach to identifying a hormone transporter or receptor is to use a radioactively labelled ligand (hormone or analogue) and attempt to purify the protein fraction(s) to which

it binds. [^{3}H]JH is available commercially as low-specific activity JH I and JH III, racemic at the epoxide position (C-10); since activity bioassays and binding tests with JHBPs both show a high degree of enantiomeric selectivity at this site in the JH molecule, the use of racemic preparations must increase the background of non-specific binding. Since the JHs are, in any case, highly prone to non-specific binding, this magnifies a chronic problem. Improved sensitivity and discrimination can be obtained through the use of higher specific activity, enantiomerically pure labelled JH, which can be prepared either by organic synthesis (Prestwich and Wawrzenczyk, 1985) or biosynthetically in cultured cockroach corpora allata for JH III (Tobe and Clarke, 1985) or *Hyalophora cecropia* male accessory glands for JH I (Ilenchuk and Davey, 1985; Palli *et al.*, 1994). Attempts to use labelled JH analogues in binding studies have been especially afflicted with problems due to non-specific binding. In a recent study with locust fat body nuclei, less than 50% of the binding of [^{3}H]methoprene was specific (competable), and with [^{3}H]pyriproxyfen no specific binding could be measured, since 100-fold excess of unlabelled ligand caused no reduction in the radioactivity bound, even though pyriproxyfen is a highly active JH mimic in the locust *in vivo* (Braun *et al.*, 1995). In the same nuclear preparations, 10*R*[^{3}H]JH III gave 80% specific binding.

For quantitative study of binding, it is necessary to separate bound from unbound ligand. Nuclei or membrane fragments may be isolated by centrifugation or filtration. For soluble proteins, the methods that have been used include precipitation of protein together with bound ligand by means of polyethylene glycol (PEG; Kovalick and Koeppe, 1983), adsorption of protein to hydroxylapatite (HAP; Goodman *et al.*, 1978; Roberts and Wyatt, 1983), adsorption of unbound ligand to dextran-coated charcoal (DCC; Engelmann, 1981a, b; Chang, 1985), and equilibrium dialysis (Park *et al.*, 1993). The different techniques may yield different results. In extracts of locust fat body, for example, the HAP assay gave higher binding values and more consistent data than DDC (Roberts and Wyatt, 1983; Shemshedini and Wilson, 1988), but a high-affinity nuclear putative receptor from *Leucophaea* fat body is reported to be detectable only by the DCC method (Engelmann *et al.*, 1987). For purified preparations of the best characterized of haemolymph JH binding proteins, the 32 kDa JHBP of *Manduca sexta*, the reported K_d values for JH I, determined by various techniques, range from 400 nM (Kramer *et al.*, 1976) down to 1 nM (Park *et al.*, 1993). When comparing the properties of different JH binding proteins, therefore, it is essential that the data have been obtained by comparable methods.

A major technical advance was the introduction of several labelled photoaffinity analogues, corresponding structurally to each of the principal naturally occurring forms of JH and containing reactive groups that link covalently to the protein to serve as tracers in analytical or purification procedures (Prestwich *et al.*, 1987, 1994). While these have been valuable

in the characterization of JH binding molecules, one must note some reservations with respect to their use in crude protein extracts. It is essential to demonstrate the specificity of binding by competition with the appropriate unlabelled ligand. The molecules detected can include JH metabolizing enzymes such as esterases and epoxidases, as well as carriers or receptors (Touhara *et al.*, 1994). High concentrations of photoaffinity ligand are required, and the method may not be sufficiently sensitive to detect JH binding proteins that occur at the low levels to be expected of nuclear receptors (Braun *et al.*, 1995). Once JH-binding components have been characterized quantitatively by other techniques, photoaffinity labelling can provide a useful tracer for protein purification and binding site identification.

3.1.2 *JH binding proteins (JHBPs) and JH uptake into target cells*

The JH circulating in insect haemolymph is chiefly bound to specific carrier proteins, which may be important in delivering the hormone to its sites of action and need to be distinguished from receptors. Studies on various insects have led to the characterization of three distinct classes of haemolymph JHBPs, and a suggestion of a fourth class (Trowell, 1992). In all of the many species of Lepidoptera that have been examined, a low molecular weight (about 30 kDa) JHBP, with a greater affinity for JH I than for JH III, has been found (Goodman and Chang, 1985; Kurata *et al.*, 1994). The low molecular weight JHBP of *Manduca sexta* has been studied in detail. Its haemolymph level is very low (20–40 $\mu g\,ml^{-1}$), and affinity chromatography has been useful in its purification (Goodman and Goodman, 1981; Park *et al.*, 1993). The cDNA has been cloned, and encodes a polypeptide of 226 amino acids that appears to represent a family of lepidopteran JHBPs, but shows no evident homology with other reported proteins (Lerro and Prestwich, 1990; Prestwich and Atkinson, 1990). The natural 10*R*,11*S* enantiomer of the hormone is bound preferentially (but not exclusively), and the K_d for 10*R*-JH I, as estimated recently by the DOC or HAP method, is 11–13 nM (Touhara *et al.*, 1993; Park *et al.*, 1993). Initial steps have been taken toward identification of amino acids involved in the JH binding site by means of photoaffinity labelling (Touhara and Prestwich, 1992).

In several insect orders, haemolymph JH carrier proteins of high molecular weight have been identified as lipophorins. Lipophorins are the principal haemolymph lipid transport lipoproteins, composed of two subunits of about 240 kDa and 80 kDa, plus 35–50% of lipid, the level of which varies with participation in the lipid transport process (Kanost *et al.*, 1990; Blacklock and Ryan, 1994). Lipophorins are major haemolymph proteins, occurring, for example, in *Leucophaea maderae* at levels up to 12 $mg\,ml^{-1}$, or 20–30% of total haemolymph protein (Engelmann, 1987), and in *Diploptera punctata*

at 4–16 mg ml^{-1}, or 10–40% of total haemolymph protein (King and Tobe, 1993). In members of the Orthoptera and Lepidoptera, such as *Locusta* and *Manduca*, in which the role of lipophorins in carrying lipid to flight muscle has been intensively studied, these proteins bind JH only non-specifically with low affinity, presumably through the lipid moiety. In insects belonging to other orders, however, where lipophorin serves as the JH transport protein, it possesses a high-affinity, enantiomerically specific JH binding site, with a preference for JH III over JH I, located on the major polypeptide subunit (de Kort and Koopmanschap, 1987a, 1989; Kindle *et al.*, 1989; Okot-Kotber and Prestwich, 1991; King and Tobe, 1992; Trowell *et al.*, 1994). The structure, JH-binding characteristics and developmental titres of the JH-binding lipophorin of *Diploptera punctata* have recently been studied and discussed in detail (King and Tobe, 1988, 1992, 1993). While the properties and lipid transport mechanism of lipophorins have been intensively investigated (Blacklock and Ryan, 1994), their dual functionality as JH carriers is not well understood.

The Orthoptera, represented chiefly by data on *Locusta migratoria*, use yet another type of JH carrier protein. Parallel separations of haemolymph proteins by density gradient centrifugation, analysed for binding of [^{3}H]JH III, showed that in *Periplaneta* and *Leptinotarsa* the JH bound almost entirely to the lipophorin fraction, whereas in *Locusta* the binding was in a different component having a higher buoyant density (de Kort and Koopmanschap, 1986). The locust haemolymph JH binding protein was identified as a 566 kDa hexamer of 77 kDa subunits, each with a JH-binding site, containing 15% of lipid and occurring at 0.6–2 mg ml^{-1}, or 1–2% of total haemolymph protein (Koopmanschap and de Kort, 1988). It favours JH III over JH I, and is strongly selective for the 10*R* enantiomer with a K_d of about 3 nM (PEG method; de Kort and Koopmanschap, 1987b). The cDNA has been cloned (R. P. Braun and G. R. Wyatt, unpublished), and the amino acid sequence shows homology with the hexamerin superfamily of arthropod haemolymph proteins (Telfer and Kunkel, 1991; Beintema *et al.*, 1994) and no homology with the JHBP of *Manduca*. The high binding specificity and affinity of this protein make it suitable for use in a quantitative competition assay for JH III (Glinka *et al.*, 1995a). In the grasshoppers, *Melanoplus bivittatus* and *M. sanguinipes*, the major haemolymph JH-binding proteins are also high molecular weight hexamers (Winder and Roberts, 1992; Ismail and Gillott, 1995b), indicating that the type of JHBP characterized from *Locusta migratoria* may occur generally among the Orthoptera.

A haemolymph protein of native M_r 500 kDa, with subunits of 88 and 79 kDa, both of which are labelled with a JH I photoaffinity analogue, has been described from diapausing larvae of the noctuid stem borer, *Busseola fusca* (Osir *et al.*, 1991). In larval and pupal (but not adult) haemolymph of *Manduca sexta*, a JHBP with 80 kDa subunits, in addition to the 32 kDa JHBP described above, has recently been reported (Touhara *et al.*, 1994).

Although more data are needed, these findings suggest that lepidopteran species may, under some circumstances, possess hexamerin-type JHBPs.

The functions of haemolymph JH carrier proteins have been discussed in previous reviews (Goodman and Chang, 1985; Trowell, 1992), and may be summarized as distribution of the hormone, protection from enzymic degradation, and delivery to cellular sites of action. That JHBP is needed for normal JH action was demonstrated in the grasshopper, *Gomphocerus rufus*, by the fact that injection of JHBP antiserum blocked oogenesis (Hartmann, 1978). Data for several species show that almost all (>99%) of the circulating JH is in the protein-bound form, and sequestration at high-affinity JHBP binding sites will help to prevent loss of hormone by non-specific, low affinity binding to lipoprotein membranes and other surfaces. Data chiefly from Lepidoptera indicate that JHBPs protect JH against degradation by non-specific esterases and epoxide hydrolase, but permit hydrolysis by JH esterase, which has a role in regulation of haemolymph JH titres (Hammock, 1985; Touhara and Prestwich, 1994). In cockroaches, however, the lipophorin JHBP inhibits JH esterase and it is suggested that changes in haemolymph JHBP level may influence JH titre (King and Tobe, 1993). Of greatest interest in relation to the topic of this review, but also least well understood, is the role of JHBPs in delivery of JH to its cellular sites of action.

In principle, the picture for binding and transport of JH is quite analogous to that for steroid and thyroid hormones in vertebrates, where the circulating plasma contains proteins with high-affinity, specific hormone-binding sites (corticosteroid-binding globulin, CBG, sex steroid-binding globulin, SHBG, and thyroid-binding globulin, TBG) as well as various lower-affinity binders (Westphal, 1970). These hormone-binding proteins are also found intracellularly, and may carry hormones from the cell surface to nuclear receptors. The hormones, like JH, are small, lipophilic molecules which can readily diffuse through cell membranes, and much evidence indicates that plasma free hormone is the immediate source for uptake into target cells. The observed rates of uptake require that the free hormone pool be rapidly replenished from protein-bound hormone (which is usually >99% of total plasma hormone), and the rate-limiting step in the uptake process may vary under different circumstances (Mendel, 1992). Measured plasma free hormone concentrations, however, are often much too low to provide for more than a very low degree of saturation of nuclear receptors, as predicted from receptor K_d values. The resolution of this paradox may be the existence of a dynamic state, in which release of hormone from serum binding protein appears to be facilitated by some interaction with the capillary or cell membrane (Pardridge, 1987). Specific membrane binding sites for CBG, SHBG and TBG have been identified on target cells and, in the case of SHBG, studied in some detail, but their physiological significance is poorly understood (Joseph, 1994). They may be involved in receptor-mediated

endocytosis. Mammalian liver has been shown to take up retinol-binding protein from plasma (Gjoen *et al.*, 1987), and recent evidence indicates a phagosome-to-cytosol pathway for exogenous antigens in macrophages (Kovacsovics-Bankowski and Rock, 1995). But this relatively slow process seems more likely to serve to provide intracellular carrier protein than as a mechanism for entry of hormone itself.

In insects, there have been no precise, quantitative studies of the uptake of JH or analogues into target cells. In view of the molecular characteristics of JH, it is likely that diffusion of free JH, replenished by dissociation from the haemolymph binding protein pool, is the mechanism of entry into cells. The facts that JHBPs have low affinities for JH analogues such as methoprene, hydroprene and pyriproxyfen (King and Tobe, 1988; Braun *et al.*, 1995), and that these compounds can act on tissues *in vitro* without added JHBP, also indicate activity of the free agonists. Haemolymph levels of free JH, however, must be very low: in *Locusta*, if the total haemolymph JH is 300 nM (Glinka and Wyatt, 1995), the concentration of JHBP binding sites is 10 μM (Koopmanschap and de Kort, 1988) and the K_d of the JHBP is 2 nM (de Kort and Koopmanschap, 1987b; Braun *et al.*, 1995), the concentration of free JH can be calculated as 6×10^{-11} M. As with the vertebrate hormones, it is difficult to account for a functional degree of saturation of receptors which may have K_d values in the nanomolar range, and some process other than simple dissociation and association as predicted by K_d values measured *in vitro* seems to be required.

Within the target cell, JHBPs may again serve to convey JH to the nucleus. In *Leucophaea* and *Diploptera*, the JH-binding lipophorins accumulate within the ovaries during oogenesis (Koeppe *et al.*, 1981a; Rayne and Koeppe, 1988; King and Tobe, 1993). In *Leucophaea* fat body, both the cytosolic and nuclear fractions contain, in addition to a putative receptor (see below), a lower-affinity JH binder which appears to correspond to the haemolymph JH-binding lipophorin (Engelmann, 1981a, 1990; Engelmann *et al.*, 1987). The presence of JH carrier proteins in fat body may be expected since they are synthesized in this tissue (Koeppe *et al.*, 1987, 1988), but the finding of the hexameric JHBP of *Locusta* within both the cytoplasmic and nuclear compartments of fat body cells, and in other organs where it is not known to be made, suggests an intracellular transport role for this protein (Braun *et al.*, 1995).

Clearly, studies are needed on the uptake of JH and analogues into target tissues, including the distribution and roles of JHBPs.

3.1.3 *Intracellular JHBPs and receptors*

In the earlier reports, it was often assumed that intracellular proteins having a specific, high-affinity binding site for JH represent receptors (e.g. Goodman and Chang, 1985), but it has become clear that non-receptor JHBPs are often

present within JH target cells. Criteria for the recognition of receptors include specific (i.e. competable), saturable, high-affinity binding and distribution correlated with biological response to JH (Koeppe and Kovalick, 1986; Riddiford, 1994). One must be aware, however, that a receptor may be present in support of a biological response which has not been detected, and that a response may be prevented by absence of an essential factor other than a receptor. Additional criteria should include affinity for JH greater than that of the carrier JHBP of the species, intranuclear localization in the presence of the hormone (though carrier JHBP is also found in nuclear preparations), and low copy number per cell. Copy number has not been emphasized in the JH literature, but in mammalian cells steroid receptors are found in the order of 10^4 molecules per cell (Yamamoto and Alberts, 1976) and thyroid hormone receptor is found in cultured cells at about 15 000 molecules per nucleus in the absence of hormone and even less after thyroid hormone exposure (Raaka and Samuels, 1981). Assuming a similar mode of action, the JH-receptor complexes will act at a relatively small number of gene loci, few copies are therefore needed, and the presence of a great excess could reduce the sensitivity of response to altered hormone level. The intracellular JH receptor is likely to belong to the known steroid/thyroid hormone receptor superfamily (Evans, 1988; Moore, 1990), which could be demonstrated by amino acid sequence, once obtained, but it must be recognized that there is as yet no direct evidence that the JH receptor is a member of this family. Ultimately, a functional test of receptor activity – the potentiation of modified cellular or subcellular activity by JH – is required.

The JH receptor has been most persistently pursued in the fat body of the cockroach, *Leucophaea maderae*. In cytosolic preparations from adult fat body, the analysis of binding curves for JH III and fractionation on DEAE-cellulose demonstrated the presence of both lower affinity (K_d 10–40 nM) and high affinity (K_d 2–5 nM) binding components (Engelmann, 1981a). The lower affinity component was found also in haemolymph and in non-JH target tissues, and is assumed to represent the lipophorin JHBP. The high affinity component, which preferentially binds 10*R*-JH III, is found in nuclear extracts only from vitellogenic fat body (adult female, or methoprene-induced male tissue) and not from non-vitellogenic tissue (penultimate instar larva or untreated adult male); the correlation of its presence with the specific response to JH is taken to be indicative of a receptor role (Engelmann *et al.*, 1987). In whole-tissue extracts, after enrichment by ammonium sulfate precipitation and DEAE cellulose chromatography, JH binding was associated with a 65 kDa band on PAGE, reducible to 35 kDa subunits. Recently, this component has been isolated by elution from gels in sufficient amount to raise antibodies in rabbits, which were then used in immunoaffinity columns to prepare more of the protein; this had a K_d of 1–2 nM and is reported as the intracellular JH receptor (Engelmann, 1990, 1995). The designation of this protein as a receptor is

based chiefly on the correlation of its presence in the fat body of last instar larvae and adults of both sexes with competence to make Vg when induced with methoprene. While this distribution does suggest a role in JH action, the fact that 2.5 g of fat body yielded sufficient of the protein to give a stained band on gels (Engelmann, 1995) indicates an abundance much greater than that of known nuclear receptors, and the nature of its role needs further examination.

Examination of the ovaries of *Leucophaea*, which are also targets for JH action (Section 3.4.2), revealed only a single binder for JH III, and this proved to be similar to the JH-binding lipophorin from the haemolymph (Kovalick and Koeppe, 1983; Koeppe *et al.*, 1984). By contrast, Engelmann (1984) detected two binding proteins in the soluble fractions from the vitellogenic ovary of *Leucophaea*. One of these binding sites has a very high-affinity for JH III (K_d approximately 1 nM), and was assumed to be a receptor protein. Only a single binding site was detected in the soluble fractions of the ovaries of *Sarcophaga*, and this was similar to the haemolymph JHBP (Van Mellaert *et al.*, 1985).

In another cockroach, *Diploptera punctata*, JH-binding proteins have been examined in brain tissue, because of evidence for a feedback effect of JH on the brain in regulating JH synthesis (King *et al.*, 1994). Specific binding of JH III was found in both cytosol and nuclear preparations, with K_d values measured as 66 and 170 nM, respectively, but competition experiments showed a higher affinity for JH II (although JH III is the natural hormone of this species). The comparatively low affinities of these proteins for JH III, relative to that of the haemolymph JHBP of the same species (2–5 nM; King and Tobe, 1988), indicate that they are not receptors, and their roles are unclear.

In the long hyaline tubules of the male accessory gland of *Melanoplus sanguinipes*, Ismail and Gillott (1994) have demonstrated a cytosolic JH-binding site with a K_d of 8.7 nM. Using photoaffinity labelling and PAGE, they found only one binding protein with M_r of 40 kDa.

The intensive investigation of JH action in the fat body of adult *Locusta migratoria* (Section 3.5) demands identification of the locust's JH receptor. An early study of binding in fat body cytosol used DEAE cellulose chromatography to isolate a protein component that bound [^{3}H]JH I with a K_d of 17 nM, competed equally by JH I or JH III and about 10-fold less by methoprene, which was regarded as a potential receptor (Roberts and Wyatt, 1983). The sedimentation constant was estimated as 12S, indicating a high molecular weight. The component was said to differ from the JH binding proteins of locust haemolymph, but the characterization of haemolymph JHBP (Roberts, 1985) may have suffered from protein degradation (see above).

Recently, JH binding in *Locusta* fat body has been reinvestigated with biologically prepared higher specific activity 10*R*-[^{3}H]JH III, which gives

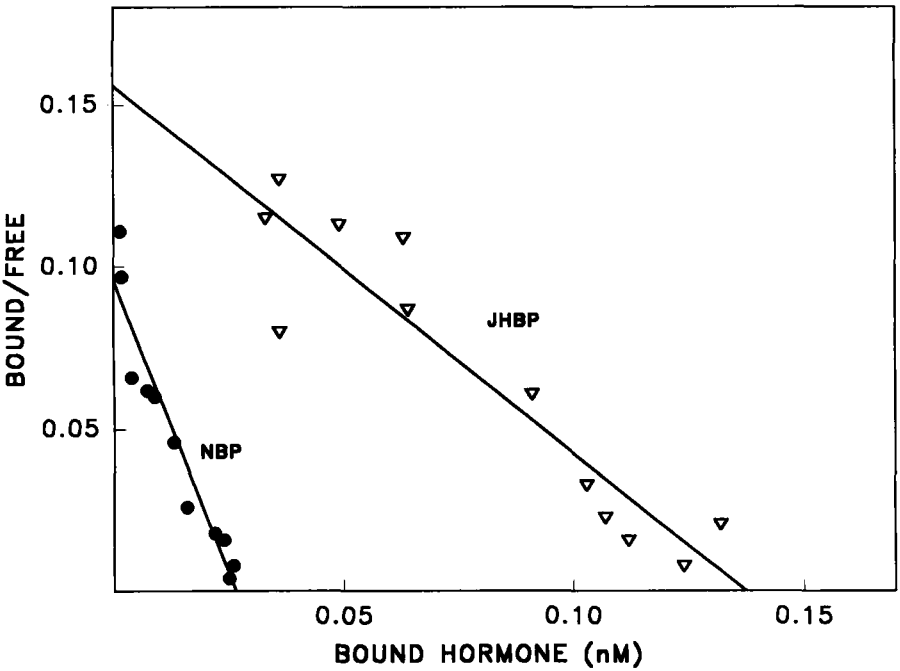


FIG. 5 Scatchard plots of JH III binding to JHBP (▽) and to putative fat body JH receptor (nuclear binding protein, NBP, •) from *Locusta migratoria*. JHBP was purified from haemolymph; the NBP fraction was a high-salt extract of adult fat body, from which JHBP had been removed by immunoprecipitation. Binding of [^{3}H]JH III was determined with hydroxyapatite. The estimated parameters are: JHBP, K_d = 1.4 nM, binding sites/nucleus = 1.5×10^6; NBP, K_d = 0.25 nM; binding sites/nucleus = 2.5×10^4. (Data of Braun *et al.*, 1995).

enhanced sensitivity and a high proportion of specific binding (Braun *et al.*, 1995). Cytosol and nuclear (high salt) extracts were fractionated on heparin-agarose columns, yielding several distinct peaks of JH-binding activity, but, by the use of an antiserum prepared against purified haemolymph JHBP, as well as photoaffinity labelling and PAGE analysis, all of the resolved components were identified as forms of JHBP, composed of 80 kDa subunits associated in different numbers and otherwise modified. The K_d for 10*R*-JH III was estimated by the HAP assay as 1.4 nM, and JH I competed about 10-fold less strongly than JH III. In the light of this evidence, the fat body cytosol fraction previously described as a potential receptor (Roberts and Wyatt, 1983) must be JHBP. After removal of JHBP from fat body nuclear extracts by exhaustive immunoprecipitation, a minor JH-binding fraction (less than 1% of total specific binding, or about 25 000 molecules per nucleus) remained which exhibited still higher affinity for JH III (K_d 0.25 nM; Fig. 5) (Braun *et al.*, 1995). This component is a reasonable candidate for the fat body JH nuclear

receptor. It was enriched in nuclear extracts by ammonium sulfate precipitation, but its very low level of occurrence renders its purification from locust fat bodies in quantities sufficient for characterization a formidable project which has so far not been undertaken.

The grasshopper, *Melanoplus bivittatus*, which is likely to use similar regulatory mechanisms, has also been used in studies on JH binding (Roberts and Jefferies, 1986). Cytoplasmic extracts of fat body yielded several JH-binding components which could be separated by chromatography and sucrose gradient centrifugation but were not further identified. Isolated grasshopper DNA had some associated protein from which two JH-binding peaks were separated by chromatography. Possibly of greatest interest, when fat bodies in culture were exposed for 3 h to [^{3}H]JH III and nuclei were then isolated, undegraded JH III could be recovered from them; of this, some 25% was bound to proteins that could be extracted with 0.3 M KCl. The JH-binding capacity of this fraction increased in amount during maturation of adult female grasshoppers to reach a maximum of about 19 000 sites per nucleus at the stage of vitellogenesis, and the K_d for racemic JH III was reported as 1.85 nM. A more recent report using electroblotting to detect JH binding proteins from *Melanoplus* fat body found several components in different size classes but did not shed further light as to their nature (Jefferies and Roberts, 1990).

Research aimed at characterization of JH receptors from tissues of larval *Drosophila*, and the embryonic Kc cell line, have been summarized by Riddiford (1994) and are of potential interest in the present context, since it is likely that a species may utilize the same or similar receptors for its pre-and post-metamorphic functions of JH. None of these proteins has been demonstrated to have receptor function, however. Of great interest is the *Methoprene-resistant (Met)* mutant, in which an 85 kDa cytosolic JH binding protein isolated from both larval fat body and adult male accessory gland had a 10-fold lower binding affinity for JH III than did the wild-type protein, suggesting that it plays a receptor role (Shemshedini and Wilson, 1990; Shemshedini *et al.*, 1990). This work is discussed further in Section 3.4.1.

Another protein recently proposed as a putative JH receptor was isolated from the larval epidermis of *Manduca sexta* (Riddiford, 1994; Palli *et al.*, 1994). After labelling with a JH photoaffinity analogue, a 29 kDa nuclear protein was isolated and partially sequenced, and the sequence data were used for isolation of a cDNA clone. Protein produced by a recombinant baculovirus appeared to bind JH I. The temporal distribution of the mRNA and protein was correlated with phases of JH activity, consistent with expectation for a JH receptor. Subsequent studies, however, using more highly purified 29 kDa protein, have shown that it does not bind labelled JH I with high-affinity. The binding studies reported (Palli *et al.*, 1994) were artifactual owing to the presence of a contaminating esterase (Charles *et al.*, 1996).

3.2 EFFECTS OF JH ON METABOLISM

3.2.1 *Respiratory metabolism*

It is self-evident that there will be metabolic consequences associated with many of the wide variety of effects which JH exerts upon its target tissues. It is thus scarcely surprising that among the effects of allatectomy detected by the early workers in the field was a decrease in O_2 consumption (Thomsen, 1949; Sägesser, 1960; de Wilde and Stegwee, 1958). Other workers noted an increased rate of O_2 consumption in muscle homogenates from allatectomized cockroaches (Samuels, 1956), and still others found only equivocal responses of tissue homogenates to extracts of CA (Ralph and Matta, 1965). It is not clear, however, that these effects are direct.

In an early experiment, Clarke and Baldwin (1960) reported that the addition of a crude extract of CA to suspensions of mitochondria from *Locusta* flight muscles or fat body increased O_2 consumption, but there is no evidence that this effect was due to JH, the concentrations of which in such extracts would have been vanishingly low. Chefurka (1978) examined the effect of JH I, JH II and JH III, as well as methoprene and some other synthetic analogues, on mitochondrial preparations from mouse liver. The compounds tested all uncoupled oxidative phosphorylation, stimulating succinate oxidation, activating ATP hydrolysis and increasing the permeability of the inner membrane of the mitochondria. But the concentrations at which these effects on a non-target tissue were maximal were of the order of 10^{-4} M, well above the JH concentration at which physiological events are optimally affected.

Similarly high concentrations were required to elicit responses from isolated mitochondria prepared from homogenates of whole larvae of *Plodia interpunctella* (Firstenberg and Silhacek, 1973). In this study, JH I at 10^{-4} or 10^{-5} M produced an inhibition of electron transport and a stimulation of succinate oxidation. Because this study and the experiments of Chefurka (1978) both used high concentrations of JH and did not explore the effects of structurally similar, but inactive compounds (such as methyl palmitate), the results may be manifestations of structural alterations in membranes which have been shown to be caused by concentrations of JH in this range (Barber *et al.*, 1981).

When cells of the *Drosophila* Kc 0% cell line (so designated because of their ability to grow in the absence of fetal bovine serum) were exposed to JH (in the fM to nM range) and their mitochondria isolated, the cytochrome oxidase activity and rate of protein synthesis of such preparations was increased by an amount proportional to the time of exposure to the hormone up to 12 hours (Stepien *et al.*, 1988). JH when applied directly to isolated mitochondria was without effect, but mitochondria exposed to post-JH, mitochondria-free supernatants exhibited an increased rate of protein

synthesis. These facts led the authors to conclude that the effect of JH on respiratory metabolism is indirect and possibly proceeds via the nucleus.

Thus, there is no doubt that removal of the CA in most insects leads to a decrease in respiratory metabolism, but there is no convincing evidence that this is the result of a direct effect of JH on mitochondria. There have yet to be done carefully controlled experiments which employ mitochondria isolated from tissues known to be targets exposed to physiological concentrations of both the hormone and an inactive analogue.

3.2.2 *Lipid metabolism*

Much evidence has accumulated that demonstrates a link between JH and lipid metabolism, and suggests a relatively direct inhibitory effect of JH on the accumulation of lipid in the fat body. The early report of Pfeiffer (1945) showing that allatectomy of the grasshopper, *Melanoplus differentialis*, was followed by accumulation of fat, eventually up to three times the normal amount, was followed by similar findings for a number of other species. The work up to about 1980 has been covered in several reviews (Wyatt, 1972; Steele, 1976, 1985; Keeley, 1978; Downer, 1985), in which the original references may be found. Steele (1985) and Downer (1985) give tables of species, including representatives of six orders, in which allatectomy was shown to be followed by whole body or fat body accumulation of lipids (see also Herman (1975b) for *Danaus plexippus*). In many instances the effects of allatectomy were reversed by implantation of active CA. These effects were observed chiefly in adult insects and were not consistently reproduced in the relatively few experiments performed with larvae. In many cases, lipid accumulation was observed over periods of many days or several weeks. Among the reports on adult insects, the only exceptions to the general inverse correlation between JH activity and lipid accumulation were in *Aedes taeniorhynchus* and *Drosophila melanogaster*, and these may reflect the precise experimental techniques used, since other reports on Diptera (including *D. melanogaster*) are in accord with the consensus.

A possible physiological meaning for the phenomenon, suggested by Downer (1985), is that phases of low JH titre, such as the early adult life of locusts or short-day induced diapause in the Colorado potato beetle, are characterized by the accumulation of lipid reserves which can provide for the energy needs of migration or diapause. With subsequent elevation of JH and onset of reproductive activity, these reserves can be utilized in vitellogenesis and other reproductive processes. In terms of mechanism, however, it is clear that the effect of JH upon fat body lipids is not secondary to the demands of the ovary, since the changes in the fat body are not prevented by ovariectomy and are found in male, as well as female insects. A repressive effect of JH upon lipid synthesis in the fat body is most clearly shown by measurements of the incorporation of label from radioactive glucose, acetate

or palmitate into lipid. In adult female *Schistocerca gregaria*, for example, allatectomy was followed by abnormal accumulation of lipid in the fat body and elevated incorporation of [^{14}C]acetate (Hill and Izatt, 1974); at the same time, incorporation of [^{3}H]leucine into fat body protein was repressed, and the authors suggest that JH has a dual effect on fat body, suppressing lipid synthesis and stimulating vitellogenic protein synthesis. A direct effect upon fat body was demonstrated by Gilbert (1967), who found that incorporation of [^{14}C]palmitate into lipid in *Leucophaea* fat body during 1 h *in vitro* was repressed by the presence of *Hyalophora cecropia* CA, whereas in a parallel incubation with ovaries incorporation into lipid was stimulated. Recently, administration of JH III or JH analogues to gregarious-phase *Schistocerca gregaria* was found to reduce fat body fresh weight and the release of diacylglycerols into the haemolymph during flight, which were interpreted as solitarization effects (Schneider *et al.*, 1995).

Virtually nothing is known of the mechanism by which JH suppresses fat body lipid synthesis. Walker and Bailey (1971) measured effects of allatectomy on a set of enzymes associated with lipogenesis in fat body of *Schistocerca gregaria*, and found especially great enhancement, paralleling the conversion of glucose to lipid, in ATP citrate lyase (which generates acetyl coenzyme A, a direct fatty acid precursor, from citrate). Isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase, which generate reducing power in the form of NADPH, were also elevated; however, these authors believed that these enzyme changes resulted from increased growth of the tissue and were not specific consequences of the withdrawal of JH.

There has been almost no recent work on this topic. In queen bumble bees (*Bombus terrestris*), both intact and ovariolectomized, Röseler and Röseler (1988) showed that application of JH I was followed by a strong, dose-dependent reduction in fat body lipid and smaller effects on glycogen reserves, which the authors suggested were an indirect consequence of stimulated vitellogenesis. In last-instar larvae of the spruce budworm *Choristoneura fumiferana*, application of methoprene or fenoxycarb was followed by fluctuations in levels of lipids and other metabolites, and fenoxycarb produced repression of both lipid synthesis from precursors and oxidation of palmitate, but no explanations were offered for these somewhat confusing data (Mulye and Gordon, 1993a, b).

Thus, the regulation of lipid synthesis represents an important area, neglected in recent research, where JH effects have been repeatedly demonstrated but the mechanism remains totally obscure. Despite suggestions to the contrary by some authors, the evidence seems persuasive of a rather direct repressive effect of JH on fat body lipid synthesis. The problem deserves re-examination. It would be enlightening to analyse the effects of JH analogues and specific inhibitors on tracer conversion, steady-state levels of intermediates and coenzymes, and critical enzyme activities, in order to

identify metabolic control points and gain understanding of their relationship to JH action.

3.3 TWO ACTIONS OF JH: PRIMING AND REGULATION

Before continuing with the evidence bearing on mechanisms of JH action at the cell and molecular level, it is helpful to establish a guiding framework. In reviewing how JH controls a wide variety of functions in the adult insect, one can perceive at least two kinds of action. JH clearly controls some specific processes, such as the initiation and rate of Vg synthesis in the fat body, the rate of protein synthesis in the male accessory gland, or the degree of patency of the ovarian follicular epithelium, and it exerts those controls in a dose-dependent and immediate way. These immediate effects can usually be demonstrated *in vitro*.

On the other hand, there is a type of action exerted by JH which requires a longer time to become evident after initial exposure to the hormone, and which is required to prepare the target cell so that the more rapid, rate-controlling actions can proceed. There are many examples of such action. Both in cockroaches, *Leucophaea*, and in locusts, after JH or analogue is applied to an adult female that has been deprived of JH by removal or destruction of the CA, a delay of more than a day is required before Vg from the fat body appears in the haemolymph, whereas JH applied to a female allatectomized after vitellogenesis had begun, or after a previous application of JH, produces a much more rapid response (Section 3.5.4.3). The follicle cells of *Rhodnius* will not shrink in response to JH I *in vitro* if they are taken from an allatectomized insect, but application of JH early after eclosion results in development of competence for an immediate response to subsequent exposure (Section 3.4.2). Accessory glands taken from allatectomized male grasshoppers require exposure *in vitro* to JH III for many hours before an increase in synthesis of the secretory proteins can be detected, whereas the effect of JH on glands removed from intact males is immediate (Section 3.4.1). In female *Schistocerca*, JH brings about the transition from defensiveness to receptivity when approached by a male. Other examples could be cited.

When the concept of dual actions of JH on the ovary was first raised (Pratt and Davey, 1972a), the developmental, preparative action was termed 'activation', and that term was used again in a recent essay which distinguished this process from the more immediate action of JH, or 'regulation' (Davey, 1994). Since the term 'activation' is widely used to describe a variety of effects, we propose to use 'priming' to describe the preparative process, and to distinguish this from regulation.

As originally conceived, priming prepared the tissue to respond later to the same hormone by regulation of an event. Thus, a prior priming exposure to JH of some hours prepares the fat body so that JH can regulate the rate

of Vg synthesis. In the follicle cell, the intracellular events by which the cell is primed can be characterized as manufacture of the appropriate cellular machinery for response to the regulatory action of JH (Section 3.4.2). JH can, however, prime cells for a response to another hormone or stimulus. In the mosquito fat body, for example, the action of JH may be confined to priming, while regulation of Vg synthesis appears to be a function of ecdysone (Section 2.1.1.9; Davey, 1994). Some of the effects of JH on behaviour, which have been described as 'modifier' effects (Truman and Riddiford, 1974; Section 2.5), and involve the development of a state of readiness for a behavioural response to a neural or neuroendocrine stimulus ('releaser' effects), appear to fall within our concept of priming.

The evidence from ultrastructural studies is relevant. In the fat body of mosquitoes, three phases can be recognized (Raikhel, 1992). During the previtellogenic period before the blood meal, characterized by high titres of JH, the nuclei enlarge and the cells proliferate organelles such as ribosomes and rough endoplasmic reticulum. The second phase is that of Vg synthesis, which is initiated by a blood meal, causing a fall in JH titre and a rise in 20HE. The organelles associated with protein synthesis continue to proliferate during the early stages of this phase, while Vg synthesis is increasing; thus, the priming and regulatory events can overlap in time. The third phase is termination, during which autophagic vacuoles degrade the protein synthetic machinery. These observations focus attention on the development of the machinery required for Vg synthesis as the target of JH in its priming role. Similar observations about the role of JH in stimulating the development of cellular machinery for Vg synthesis have been made for the cockroach, *Leucophaea*, and the locust (Section 3.5.2), in which JH also provides the stimulus regulating Vg synthesis itself. Ultrastructural studies on male accessory glands, while not so detailed as those for the fat body, also show that among the roles of JH is proliferation of the machinery for protein synthesis (Section 2.3.3).

In addition to proliferation of the general apparatus for protein synthesis, however, it is clear that the processes covered by our concept of priming include the provision of specific factors that make possible subsequent specific regulatory responses. In the ovarian follicle cell, for example, these include synthesis of membrane JH receptor and Na^+/K^+ ATPase. In the fat body, they include factors required to initiate transcription of *Vg* and other JH target genes. The priming action of JH is seen to be acting via the nucleus, putting in place the general and specific cellular machinery through which JH (or other hormones) will act to regulate various processes.

The second, or regulatory, action of JH, as will become clear in the following sections of this review, can involve at least two classes of action, probably utilizing different receptors. One acts at the level of the membrane, as in the control of patency in the follicular epithelium, and one acts through the nucleus, regulating, for example Vg synthesis, by activation of specific genes.

The proposition that there are two clear roles of JH in the control of various adult tissues has some potential consequences. The priming function may have receptors different from those that mediate the eventual regulatory response. This is perhaps clearest in a system like the follicle cell, where the receptors are likely to be located in different cellular compartments. In the control of Vg synthesis, on the other hand, priming and regulation may be at least partly parallel events, both likely to stem from a common receptor located in the nucleus but differently expressed as a result of different states of the intracellular amplification system. We will return to this analysis of the actions of JH at the end of this review.

3.4 ACTIONS OF JH AT THE CELL MEMBRANE

While many actions of JH are considered to be focused in the nucleus (Riddiford, 1994; Jones, 1995; Section 3.5), it has become clear that some of the actions are mediated by events in the cell membrane. Thus far, two tissues, the male accessory glands and the ovarian follicle cells, have been demonstrated to respond to JH partly via the cell membrane.

3.4.1 *The male accessory glands*

There is evidence which indicates that the effect of JH on protein synthesis in the accessory glands involves some form of transmembrane signalling. In *Drosophila*, Yamamoto *et al.* (1988) showed that in male accessory glands incubated *in vitro* there is an increase in the synthesis of both RNA and protein when the glands were removed from males after copulation. JH III caused an increase in protein synthesis when added to the incubation medium in concentrations ranging from 0.01 to 1 nM. Ca^{++} was essential for this effect: in the absence of added Ca^{++} the effect of JH III was reduced, and when Ca^{++} was removed by chelation, the effect of JH III was abolished. The required level of Ca^{++} in the medium was 0.1 mM, and higher concentrations had no effect on the JH response of the glands but could increase protein synthesis without added JH. In the absence of added Ca^{++}, the iontophore A23187 stimulated protein synthesis. The presence of Co^{++}, an antagonist of Ca^{++}, in the medium reduced the effect of added Ca^{++}. These results argue for the involvement of Ca^{++} in the stimulation of protein synthesis by JH.

The same authors brought forward evidence which demonstrates that protein kinase C is also involved in the response. The phorbol ester, PDBU, which is known to stimulate protein kinase C, mimicked the action of JH by causing an increase in protein synthesis in the *in vitro* system provided that Ca^{++} was present in the medium. The addition of cyclic nucleotides involved in other signalling pathways was without effect. When normal males were exposed to methoprene, their accessory glands showed an enhanced

level of protein synthesis when placed *in vitro* one hour later, but when males of the mutant *tur*, defective in kinase C activity, were similarly treated, there was no increase in protein synthesis.

These results show a striking similarity to those obtained on the ovarian follicle cells (Section 3.4.2) in that the action of JH can be mimicked by activators of protein kinase C. While there is good reason to suggest that the action of JH on the accessory glands may involve a membrane receptor, there is no direct evidence from these experiments that the primary site of action of JH is at the membrane. The experiments on the follicle cells, however, were conducted on membrane preparations free of cytosol.

Preliminary results (Sevala *et al.*, 1995) have demonstrated the existence of binding proteins for photoaffinity ligands in membrane preparations from male accessory glands of both *Rhodnius* and *Locusta*. Membrane preparations of the transparent accessory gland of male *Rhodnius* specifically bind the photoaffinity analogue of JH I, [^{3}H]-EBDA, and the binding is associated with a peptide of approximately 51 kDa on denaturing gels. In *Locusta*, labelling with [^{3}H]-EFDA reveals a 40 kDa peptide with binding competable by JH III. In neither case is there evidence to link these binding proteins to any function.

Other results, however, demonstrate that the situation is far from simple. Shemshedini *et al.* (1990) have identified specific and saturable binding of JH III in cytosol preparations from the male accessory glands of *Drosophila*, with a K_d of 6.7 nM. Using the photoaffinity ligand [^{3}H]-EFDA, they found specific labelling to be associated with an 85 kDa peptide. The mutant *Met* is highly resistant to the effects of JH and methoprene, and this is reflected in a failure of JH III to stimulate protein synthesis in the accessory glands of the mutant and a greatly reduced affinity for specific JH binding in cytosolic fractions from such glands. There is thus a correlation between the presence of the binding protein and the ability of JH to stimulate protein synthesis in the gland. While this does not constitute proof of a functional link, it strongly suggests the involvement of a cytosolic JH binder in the stimulation of protein synthesis. In more recent work (Shemshedini and Wilson, 1993), RNA synthesis in the accessory gland was also stimulated by JH III, but inhibition with actinomycin D did not cause concurrent reduction of protein synthesis, as would be expected if protein synthesis were regulated at the level of transcription. From this, the authors suggest a dual effect of JH in the male accessory gland, the stimulation of RNA and protein synthesis being independent effects of the hormone. While a dual effect via membrane and intracellular receptors would be consistent with other evidence on this gland, it is not clear that the inhibition of RNA synthesis achieved by Shemshedini and Wilson (1993) with actinomycin D (which preferentially affects transcription of rRNA) was sufficiently complete to justify dissociating the control of protein synthesis from transcription.

A binding protein for JH III has also been described in cytosolic fractions

prepared from the long hyaline tubules (LHT) of the accessory gland of *Melanoplus sanguinipes* (Ismail and Gillott, 1994). Cytosol binds JH III in a specific and saturable fashion, with a K_d of 8.7 nM. By exposing blots of SDS-PAGE gels to [^{3}H]JH III, or by fluorography of gels prepared from cytosol labelled with the photoaffinity ligand, [^{3}H]-EFDA, the binding was shown to be uniquely associated with a 40 kDa peptide. The functional significance of this binding protein is not yet clear, although a good deal is known about the effects of JH on the glands of *Melanoplus* (Section 2.3.3).

The accessory glands of male *Locusta* have recently been examined for JH binding proteins (Braun and Wyatt, 1995). While abundant binding for [^{3}H]10*R*-JH III was found in both cytosolic and nuclear fractions, this was traced to a protein identical to the hexameric JHBP found in the haemolymph. Observations on the time-course of stimulation of protein synthesis in the locust male accessory gland by a JH analogue show that the response is much more rapid than that in the fat body of the same animal (Fig. 6), suggesting that the hormonal stimulus may be mediated by different mechanisms in the two tissues. This could be explained if the accessory gland uses a membrane receptor, while the activation in the fat body proceeds via the nucleus. The evidence thus far available is too fragmentary to draw any firm conclusions about the pathway(s) involved in the control of protein synthesis by JH in the accessory glands of male insects. There is strong circumstantial evidence for the involvement of both transmembrane signalling and internal receptors. These need not be mutually exclusive mechanisms, nor, equally, need they be parallel. The evidence fits the model outlined in Section 3.3, whereby JH is envisaged as acting first as a primer, preparing the target cell to respond subsequently to the same hormone as a regulator of a specific function. There is evidence in both *Melanoplus* and *Locusta* that there are two phases in the action of JH on the accessory glands (Section 2.3.3).

3.4.2 *Ovarian follicle cells*

In most insects, the Vg in the haemolymph reaches the oocyte surface by passing through large spaces which appear between the cells of the follicular epithelium (Section 2.2.1.3; Fig. 2). In *Rhodnius prolixus*, the appearance of these spaces, a condition termed patency, was suggested to be under the control of JH (Pratt and Davey, 1972a). It was then demonstrated that JH reversibly induced patency in ovaries incubated *in vitro*, and that the development of patency was rapid, reversible and unaffected by inhibitors of macromolecular synthesis (Davey and Huebner, 1974; Abu-Hakima and Davey, 1977a).

The appearance of the intercellular spaces is primarily the result of a reduction in volume of the follicular cells (Abu-Hakima and Davey, 1977b).

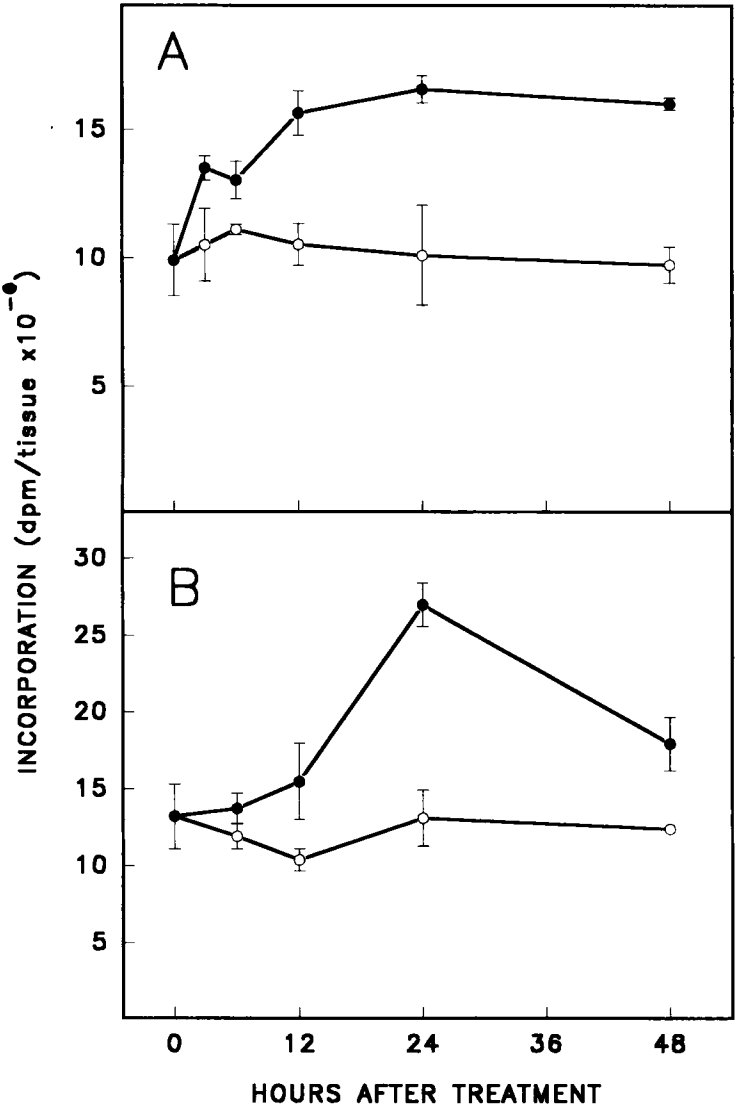


FIG. 6 Time-course of protein synthesis stimulation by a JH analogue in male accessory gland and female fat body of *Locusta*. Adult locusts, previously treated with precocene to destroy the CA, were topically treated with either 10 μg pyriproxyfen in acetone (●) or acetone vector (○). At intervals, accessory glands from males (A) and fat bodies from females (B) were removed, incubated for 3 h with [^{3}H]leucine, and incorporation was measured into TCA-precipitable protein. Values are means ± SEM (n = 3). (Braun and Wyatt, 1995).

Ouabain inhibited the action of JH (Abu-Hakima and Davey, 1979), and exposing membrane preparations from vitellogenic follicle cells to JH *in vitro* increased the activity of Na^+/K^+ ATPase (Ilenchuk and Davey, 1982, 1983). This was due to the activation of a novel form of the enzyme, as defined by the binding characteristics of radio-labelled ouabain (Ilenchuk and Davey, 1983). Experiments with inhibitors and activators of protein kinase C indicated that this enzyme is involved in the activation of the ATPase (Sevala and Davey, 1989, 1990). Exposure of membrane preparations from *Rhodnius* follicle cells to JH I also results in the phosphorylation of a 100 kDa protein, a process which is inhibited by ouabain and depends on activity of protein kinase C. It has been suggested that the 100 kDa protein may represent the α-subunit of the Na^+/K^+ ATPase (Sevala and Davey, 1993).

In this system, it is clear that JH I is acting at the level of the membrane. JH I binds in a specific and saturable fashion to membrane preparations from *Rhodnius* follicle cells with a K_d of 6.54 nM (Ilenchuk and Davey, 1985). Neither JH II nor JH III exhibits biological activity or any competition for the binding site (Ilenchuk and Davey, 1987b). A conceptual model for this action of JH on the follicle cell membrane is outlined in Fig. 7.

A similar system operates in the follicle cells of *Locusta migratoria*, but uses JH III rather than JH I to induce patency via shrinkage of the follicle cells. This action is also inhibited by ouabain (Davey *et al.*, 1993). By using [^{3}H]EFDA, a photoaffinity analogue of JH III, a 35 kDa peptide has been identified on denaturing gels prepared from solubilized membranes from locust follicle cells (Fig. 8). The binding of EFDA is blocked by prior exposure of the membranes to JH III, and the membrane preparations bind [^{3}H]JH III in a specific and saturable fashion (K_d = 3.68 nM). In contrast to *Rhodnius* follicle cells, those of the locust fail to respond to JH I, but they undergo some reduction in volume when exposed to JH II. EBDA, the photoaffinity analogue of JH I, exhibits no specific binding to the locust membrane preparations, while EHDA, the analogue of JH II, binds specifically to a 35 kDa peptide. JH I does not compete for the JH III binding site on locust membrane preparations (Davey *et al.*, 1994; Sevala *et al.*, 1995).

The specificity of the membrane binding in the two species, whereby *Rhodnius* follicle cells bind only JH I, but not JH II or JH III, while the locust cells bind JH II and III, but not JH I, is provocative. These variants of JH differ only in the side chains, where JH I is 7,11-diethyl, JH II is 7-methyl,11-ethyl and JH III is 7,11-dimethyl. This suggests that the *Rhodnius* receptor requires at least one ethyl group and the *Locusta* receptor requires at least one methyl group in these two positions. This great specificity is unlike that found for other JH binding proteins, where some degree of competition is exhibited by all three forms of JH. It is possible that any membrane receptor is qualitatively different from other binding proteins. However, these studies have all been done on follicle cell membrane

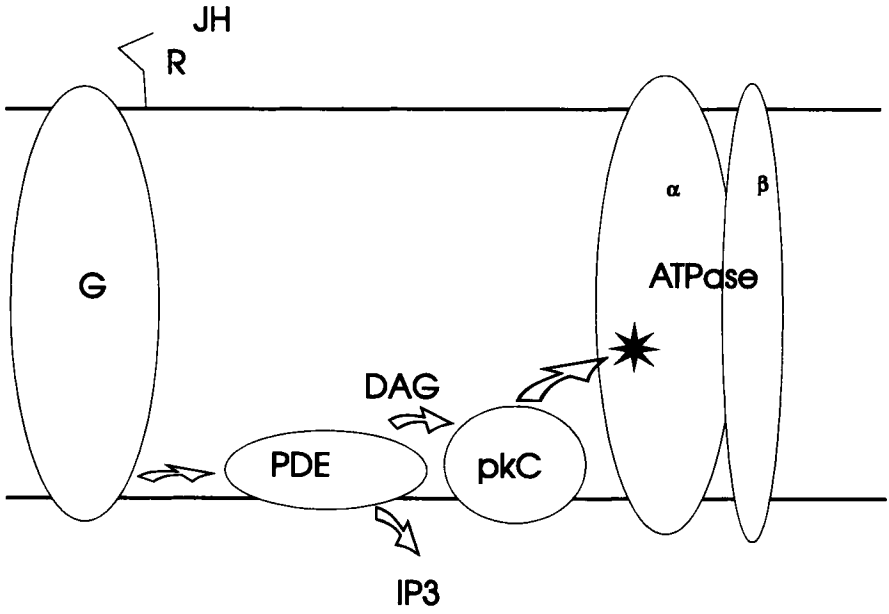
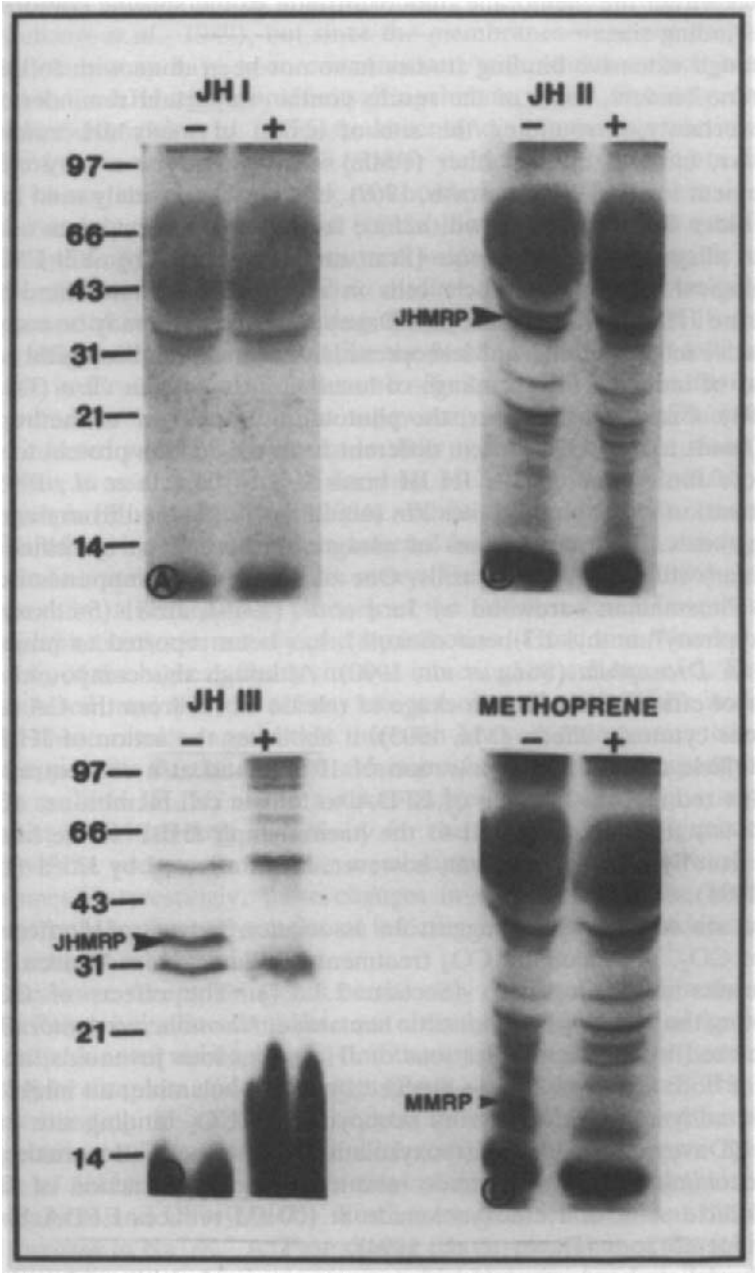


FIG. 7 Proposed conceptual model for the action of JH on the membrane of the ovarian follicle cell. The arrival of JH at the receptor site (R) on the outer surface of the membrane initiates a cascade involving a G protein (G), with which the receptor site may be closely associated. Protein kinase C (pkC) is activated through phosphodiesterase (PDE) and diacylglycerol (DAG) and phosphorylates the α-subunit of the Na^+K^+ ATPase, activating the enzyme. The phosphodiesterase may also provide a link to as yet undescribed events in the cytoplasm through the phosphoinositide system. The JH binding protein, protein kinase C, and the Na^+K^+ ATPase are the only elements for which experimental evidence of their involvement exists.

FIG. 8 Photoaffinity labelling of putative JH receptor from *Locusta* ovarian follicle cell membranes. Portions of membrane protein were incubated with the following photoaffinity analogues: (A) [^{3}H]EBDA (analogue of JH I); (B) [^{3}H]EHDA (JH II); (C) [^{3}H]EFDA (JH III); (D) [^{3}H]MDK (methoprene). Each reaction was incubated with (+) and without (−) excess unlabelled JH I, JH II, JH III, or methoprene, respectively. Proteins were resolved by SDS-PAGE and labelled components were detected by fluorography. The photoaffinity analogues labelled several proteins, but specific labelling is that which is abolished in the presence of competing hormone. There is no specific labelling by the analogue of JH I, but the analogues of both JH II and JH III specifically label a 35 kDa protein (JHMRP). By contrast, the analogue of methoprene specifically labels a 17 kDa protein (MMRP). (Reprinted with permission from Sevala *et al.*, 1995.)


JH I
JH II
JH III
METHOPRENE
97
66
43
31
21
14
JHMRP
MMRP

preparations rather than on solubilized protein, and it is possible that its situation within the membrane may contribute to the specific conformation of the binding site.

Although extensive binding studies have not been done with follicle cell membrane binders, some of the results contain important reminders about the uncertainty surrounding the site of action of many JH mimics. In *Rhodnius*, farnesyl methyl ether (FME) is known to be a very effective replacement for JH (Wigglesworth, 1969), and has been widely used in some of the older literature. FME will induce full egg production when administered to allatectomized *Rhodnius* (Pratt and Davey, 1972b). Yet FME has no biological activity on follicle cells *in vitro*, and does not bind to the membrane JH binder (Ilenchuk and Davey, 1987b). FME may be converted to an active molecule *in vivo*. Methoprene, by contrast, has biological activity in terms of inducing the shrinkage of locust follicle cells *in vitro* (Davey *et al.*, 1993). Curiously, however, the photoaffinity analogue of methoprene, MDK, binds to a 17 kDa protein different from the 35 kDa protein to which the photoaffinity analogue of JH III binds (Fig. 8; Sevala *et al.*, 1995).

Information about binding sites can sometimes be gleaned from the effects of antagonists. Two new classes of antagonists have been identified from preliminary studies on follicle cells. One of a series of compounds isolated from a Panamanian hardwood by Jurd *et al.* (1979), J2581 (5-ethoxy-6-(4-methoxyphenyl)methyl-1,3-benzodioxole), has been reported to inhibit YP uptake in *Drosophila* (Song *et al.*, 1990). Although this compound has a number of effects, including blockage of release of JH from the CA as well as various cytotoxic effects (Ma, 1993), it abolishes the action of JH III on locust follicle cells at a concentration of 10 nM, and at a concentration of 100 nM it reduces the binding of EFDA to follicle cell membranes (Davey *et al.*, 1994). Binding of JH III to the haemolymph JHBP of the Mediterranean fruit fly, *Ceratitis capitata*, however, is not affected by J2581 (Chang *et al.*, 1991).

There are reports which suggest an association between JH effects and those of CO_2. For example, CO_2 treatment of allatectomized queen honey bees results in vitellogenesis (Section 2.1.1.7). The effects of CO_2 in stimulating the ecdysis of the parasitic nematode, *Haemonchus contortus*, can be mimicked by high concentrations of JH and various juvenoids, and the effects of both CO_2 and JH are blocked by ethoxyzolamide, an inhibitor of carbonic anhydrase which acts by occupying the CO_2 binding site on the enzyme (Davey *et al.*, 1983). Ethoxyzolamide and other sulfonamides such as acetozolamide and dansylamide inhibit the biological action of JH on locust follicle cells, and ethoxyzolamide at 100 nM reduces EFDA binding to their membranes (Davey *et al.*, 1994).

A JH-mediated induction of patency appears to be fairly widespread in insects. Even among the Diptera, where Vg or YP synthesis is principally controlled by 20HE, JH dependent patency has been detected (see reviews

by Kelly, 1994 and Davey, 1994). Microsomal preparations from the ovary of *Sarcophaga bullata* exhibit specific binding of JH III of rather low affinity (Van Mellaert *et al.*, 1989), but since the membranes were not prepared in high salt, they may have been contaminated with vitellin, which has been reported to bind JH with low affinity (Engelmann, 1984). Similarly, an early attempt by Schmialek *et al.* (1975) to detect binding in various fractions from extracts of the ovaries of *Tenebrio molitor* yielded results which are difficult to place in our current framework of knowledge.

More recently, Webb and Hurd (1995a) have demonstrated specific and saturable binding of JH III to microsomal preparations of vitellogenic follicles from the mealworm beetle, *Tenebrio molitor*. Scatchard analysis suggested the presence of two sites of differing affinity, one with a K_d of 10 nM, and a lower affinity site at 400 nM. Unlike the binding site in locust follicle cells, both sites in *T. molitor* exhibited some affinity for JH I. *T. molitor* acts as an intermediate host for the rat tapeworm, *Hymenolepis diminuta*, and beetles infected with the metacestode show reduced egg production (Hurd, 1993). This is associated with an increase of 70% in the titre of Vg in the haemolymph (Hurd and Arme, 1984). While there is a small reduction in synthesis of Vg by the fat body in infected beetles, there is a much greater reduction in uptake of Vg into the ovary (Hurd and Arme, 1986). Infected beetles exhibit a reduction in patency of the follicle cells. JH synthesis and haemolymph titres are not affected by infection (Hurd and Weaver, 1987), but the K_d of the higher affinity site was increased by a factor of 5. The B_{max} was unaltered, and the low affinity site was not affected (Webb and Hurd, 1995b). These results suggest that infection of the beetle causes the release from the parasite or the host of a factor which alters the capacity of the receptor to bind JH. If, as the authors suggest, the factor is a competitive antagonist of JH binding, its affinity for the binder must be great enough to survive the preparation of the membranes, which involves washing the microsomes. Interestingly, these changes in the affinity of the binder are apparent only in the early stages of infection: as the metacestode matures, the K_d returns to normal.

A membrane receptor may not be the only receptor for JH in the follicle cells. In *Rhodnius*, allatectomy does not entirely abolish growth of the oocytes: a few follicles enter vitellogenesis, and these follicles grow slowly, eventually maturing as complete and viable eggs (Pratt and Davey, 1972a). The follicle cells surrounding the vitellogenic oocytes in such allatectomized females are unresponsive to JH: apparently, follicle cells which have grown up in the absence of JH lack the capacity to respond to JH by reducing their volume (Abu-Hakima and Davey, 1975). Further analysis revealed that there was a sharp increase in Na^+/K^+ ATPase during the development of the follicle cell just prior to the appearance of patency, which did not occur in allatectomized insects. Similarly, the JH binding capacity of cell membranes from previtellogenic follicles was lower in allatectomized insects than in normal insects.

When JH I was applied 2 days after allatectomized females were fed, more follicles entered vitellogenesis, but neither the JH-sensitive Na^+/K^+ ATPase nor the JH binding site exhibited a marked increase in membranes prepared from such follicles. On the other hand, when the JH I was applied earlier, 2 days after emergence (8 days before feeding), the levels of both the JH sensitive Na^+/K^+ ATPase and the binding protein returned to normal (Ilenchuk and Davey, 1987a). Although the evidence is indirect, it strongly suggests that exposure of the follicle cells to JH I early in their development acts to initiate the synthesis and/or insertion into the membrane of both the receptor for JH I and one of the effector molecules. This provisional conclusion argues for the existence of another receptor for JH in the follicle cells, likely in the nucleus. Webb and Hurd (1995b) provide some evidence for JH binding to proteins in nuclear and cytosolic preparations for the ovary of *T. molitor*, although these binding sites are not characterized. These early, developmental events which prepare the cells for a specific, later response are an example of the priming action of JH, while the later response via a membrane receptor is a regulatory action (Section 3.3).

A reduction in cell volume is not the only event that characterizes the development of patency; modifications in cell junctions and other changes are also important (Huebner and Injeyan, 1980, 1981). In addition, of course, the events in the membrane will give rise to changes in the cytoplasm as well. Little is known about the involvement of JH in such events. Preliminary observations by one of us (K.G.D.), using the Ca^{++}-activated fluorescent dye Fluo 2 and confocal microscopy, suggest that exposure of locust follicle cells to JH results in a rapid and reversible decrease in free Ca^{++} in the cells. These observations accord well with the recent demonstration of a JH-dependent increase in transcripts coding for the Ca^{++}-binding protein, calmodulin, during development of the ovary in *Blattella germanica* (Iyengar and Kunkel, 1995).

Other sorts of molecular events in the developing follicle cells have been reported. The follicle cells of many species become amitotically binucleate (Wigglesworth, 1972), and in the cockroach, *Leucophaea maderae*, there is a JH-mediated increase in DNA synthesis in the follicle cells, leading to an increase in DNA content (Section 3.5.1). How this may be related to the developmental events in the follicle cells of *Rhodnius* is unclear, but the effect of JH on DNA replication suggests the existence of an intracellular receptor for JH. As pointed out in Section 3.1.3, soluble JH binding proteins, including a putative receptor, have been identified in the ovary of *Leucophaea* but there is no evidence as to their functions.

3.5 RESPONSES TO JH INVOLVING NUCLEAR ACTIVITY

In contrast to the responses to JH which result from direct action of the hormone at the cell membrane, discussed in Section 3.4, many of the known

consequences of JH action involve modulation of gene expression and so can be described as nuclear effects. Research in this area has focused on tissues in which major biochemical processes – such as the synthesis of Vg in fat body – are evoked by JH, and we shall review what is known about how the hormone is involved in preparing the cells for these responses, as well as in the regulation of the responses themselves.

3.5.1 *DNA replication and polyploidy*

As a part of the preparation of cells for intensive gene expression, JH can bring about marked changes in the nucleus. In *Locusta*, the previtellogenic development of the fat body involves nuclear enlargement (Lauverjat, 1977; Couble *et al.*, 1979; Irvine and Brasch, 1981), with a 3-fold increase in volume during the first 13 days of adult life in both sexes, although throughout the process the nuclei were smaller in males than in females (Jensen and Brasch, 1985). Nuclear growth was prevented by application of precocene and restored with methoprene. Also observed, and also JH-dependent, were increases in heterochromatic masses, nucleoli and various nuclear inclusions, as well as developmental changes in the nuclear matrix (Jensen and Brasch, 1985). These changes resemble those seen in other animal tissues when exposed to conditions, including steroid hormones, that stimulate DNA replication and transcription (Brasch and Ochs, 1995).

An important aspect of this nuclear development is DNA replication and increase in ploidy. Polyploidy – the existence of more than two chromosome sets within a nucleus – is common in differentiated insect tissues, where it serves to produce more gene copies without the need for mitosis. While there are a few well-documented instances of selective gene amplification in insect development, such as the chorion genes of *Drosophila* (Kafatos *et al.*, 1985) and the DNA puffs of *Rhynchosciara* (Glover *et al.*, 1982), the less efficient mechanism of replicating the entire genome is far more common. In larval insects, tissues destined for breakdown in metamorphosis commonly become polyploid during the ecdysteroid-induced moulting cycle, while imaginal disks and most tissues that will survive to the adult stage undergo mitosis and remain diploid. In the adult, on the other hand, DNA replication and increase in ploidy have been shown to be stimulated by JH in the fat body and ovarian follicular epithelium of several species.

In the fat body of *Locusta migratoria*, the previtellogenic maturation of the cells is accompanied by a doubling of DNA content (Chen *et al.*, 1979) and a rise in ploidy in the female from chiefly 4C on day 1 to chiefly 8C on day 8, with some further increase thereafter (Nair *et al.*, 1981a; Irvine and Brasch, 1981). This was prevented by allatectomy, and both thymidine incorporation and ploidy increase could be induced by application of methoprene. It is of interest to note that male fat body, which does not produce Vg, also underwent JH-dependent DNA replication, but with a more

rapid response to methoprene and a lower eventual ploidy level than the female tissue. In the desert locust, *Schistocerca gregaria*, application of hydroprene to instar IV precocious adults produced by exposure to precocene was followed by increased ploidy in both the ovarian follicle and the fat body (Nair *et al.*, 1981b). Normal adults of *S. gregaria* also exhibit ploidy increase during maturation, but the role of JH was not reported (Kooman and Nair, 1982).

In the mosquito, *Aedes aegypti*, nuclear enlargement (doubling of diameter) is reported during the previtellogenic phase, the first 3 days after eclosion (Raikhel and Lea, 1983). The fat body cells undergo ploidy increase both before and after the blood meal, up to 8n (Dittmann *et al.*, 1989). In abdomens ligated immediately after emergence, this was much reduced (but not totally blocked), and was fully restored after application of JH III or methoprene. An effect of JH on polyploidization in adult fat body has also been reported in the lady beetle, *Coccinella septempunctata* (Quan and Chen, 1983). Despite the extensive studies on the role of JH in vitellogenesis in *Leucophaea maderae*, there seems to be no report on DNA levels in the fat body. In another cockroach, *Blaberus discoidalis*, it is reported that DNA levels do not increase in the adult fat body (Mannix and Keeley, 1980).

The stimulation of DNA replication by JH in the follicular epithelium surrounding the terminal oocytes of *Leucophaea maderae* has been studied by Koeppe and coworkers. After decapitation of 1-day old adult females, injection of JH I stimulated enlargement of the cells and their nuclei, without increasing cell number (Koeppe *et al.*, 1980a). The incorporation of [^{3}H]thymidine into DNA rose after mating in correlation with the increase in haemolymph titre of JH III; after decapitation it dropped sharply and could be stimulated within 24 h by administration of JH (Koeppe *et al.*, 1980b; Koeppe and Wellman, 1980). Follicular thymidine kinase activity was found to rise in parallel with thymidine incorporation in response to JH, and this rise was blocked by actinomycin D and is therefore taken to involve transcription (LaPointe and Koeppe, 1984). After mating, the follicle cells showed a steep increase in DNA content and ploidy level (assayed by microspectrophotometry), eventually to 16- and 32-ploid; this, too, was prevented by decapitation and provoked by injection of JH III (LaPointe *et al.*, 1985). Protein synthesis in ovaries from virgin females was stimulated by JH I during culture *in vitro*, which proves that the hormone influences the follicles directly. In the final choriogenic phase of oogenesis, DNA replication ceased while protein synthesis rose steeply and was no longer prevented by decapitation (Koeppe *et al.*, 1981b). Thus, during the previtellogenic and vitellogenic stages, the follicular cells undergo JH-stimulated development based on nuclear activity, in preparation for the final, JH-independent synthesis of the proteins of the chorion.

From these examples, it can be concluded that increased genome copy number can be brought about by JH in several tissues of adult insects. Much

remains unexplored, however. It should be noted that the incorporation of thymidine, mediated by thymidine kinase, represents a 'salvage pathway' and not the main pathway of *de novo* DNA synthesis. Although it has been shown that the JH-stimulated increase in thymidine kinase is blocked by actinomycin D, it is not known whether gene transcription is essential for the increase in DNA level and ploidy. Also untested is the question whether DNA replication is a prerequisite for JH induction of genes such as *Vg*, or whether it merely provides for a high level of expression by making available more gene copies. As a part of the preparation of cells for active expression of specific genes, JH-stimulated DNA replication can be described as an aspect of priming (Section 3.3).

3.5.2 *The protein-synthesizing apparatus*

During metamorphosis the chief role of the fat body is the storage and release of nutrient reserves – lipids, glycogen and protein – in support of morphogenetic processes occurring without food intake, and at adult eclosion, the fat body principally retains the character of a storage organ. To prepare for reproduction, it must reconstruct organelles to provide for the massive synthesis of Vgs and other proteins needed for oogenesis. This previtellogenic proliferation of the fat body protein-synthesizing apparatus has been found in several species to depend on JH. The changes typically involve reduction in cytoplasmic lipid droplets, glycogen rosettes and in some species protein granules, accompanied by proliferation of rough endoplasmic reticulum to form arrays of ribosome-studded cisternae, the appearance of Golgi complexes, and increase in number and size of mitochondria. A similar ultrastructural picture has been reported in *Leptinotarsa* (de Loof and Lagasse, 1972), *Nauphoeta* (Wuest, 1978), *Calliphora* (Thomsen and Thomsen, 1974) and *Aedes* (Raikhel and Lea, 1983), and has been thoroughly described in *Locusta* (Lauverjat, 1977; Couble *et al.*, 1979). In *Locusta*, allatectomy completely prevents these changes and leads, instead, to further increase in fat droplets and diminution of cytoplasm, but this condition is rapidly reversed and restored to normal by implantation of CA or treatment with methoprene. During the subsequent vitellogenic phase, when the fat body is actively engaged in synthesis of Vg, the build-up of protein-synthesizing equipment may continue, under stimulation by JH in many insects, or 20HE in the Diptera (Section 2.1.1).

In *Leucophaea maderae*, these processes have been studied by biochemical methods. Polysome/microsome profiles from fat body of vitellogenic females showed a high-density microsomal peak, composed of ribosome-studded vesicles, differing from the microsomal peak obtained from non-vitellogenic tissue (Engelmann, 1974; Engelmann and Barajas, 1975). The incorporation of [^{14}C]choline or ^{32}P into phospholipids in both smooth and rough microsomal fractions increased sharply after application of JH III to

allatectomized females (della-Cioppa and Engelmann, 1980, 1984a). In further experiments it was found that in secondary induction, resulting from a second dose of JH III after 12 days, when the effect of the primary treatment had decayed, the stimulation of microsomal phospholipid synthesis was much accelerated (della-Cioppa and Engelmann, 1984b). Although the authors suggest that the 'proliferation of ER in *L. maderae* fat body is temporally coupled to Vg synthesis, and both phenomena are coordinately regulated by JH' (della-Cioppa and Engelmann, 1984b), the data indicate stimulation of fat body phospholipid synthesis about 24 h earlier than the appearance of labelled Vg in the haemolymph, after both primary and secondary JH treatment. It is of interest that JH stimulates phospholipid synthesis only in vitellogenic fat body (adult female or adult male with a high dose of JH), and not in larval fat body or other tissues. Clearly, ER proliferation, involving new phospholipid synthesis, is an essential preparation for fat body Vg production, which continues during Vg synthesis, and is needed for translation of secretory polypeptides, post-translational modification and export of the protein. As such, it may be described as an aspect of priming (Section 3.3).

3.5.3 *Histolysis and regeneration*

The JH-regulated involution and regeneration of flight muscles in certain species of insects illustrates another type of major structural alteration of a tissue controlled by this hormone, as described in Section 2.4. The complete and permanent loss of the flight muscles that takes place after the dispersal phase of adult life in various species of insects is clearly an example of programmed cell death (apoptosis), a process involving a programme of gene activation that is well known in animal development and is currently a topic of active research (Ellis *et al.*, 1991; White *et al.*, 1994). Programmed cell death can be triggered by a variety of stimuli, and for adult flight muscles in several insect groups the signal is apparently a rising titre of JH. The increase in ubiquitin-conjugated proteins observed in pea aphid flight muscles undergoing JH-induced breakdown (Section 2.4.3) is consistent with mechanisms known for other systems. Ubiquitin is a highly conserved, low-molecular-weight protein that serves to mark proteins for degradation by ubiquitin-dependent proteases (Finlay and Chau, 1991).

The reversible flight muscle atrophy found in scolytid beetles and *Leptinotarsa* is of exceptional interest because nuclei and other structures are maintained intact to support later regeneration of the muscles to a functional state under the stimulus of changing hormonal milieu. It is also of interest, in relation to hormonal mechanisms, that muscle atrophy is apparently induced by rising JH titre in one group of beetles (Scolytidae) and by falling JH in another (e.g. *Leptinotarsa*), as required by their respective life cycles. While some changes in muscle enzyme activities have been described, almost

nothing is known as to how they are linked to JH action. The molecular aspects of reversible muscle degeneration, including the precise role of JH, will be rewarding to study.

Another insect system, unrelated to JH, in which muscle degeneration has been studied in detail, may afford some insight into these processes. In silk moths and sphinx moths the abdominal intersegmental muscles degenerate immediately after adult eclosion. In the saturniid silk moth, *Antheraea pernyi*, two stages can be recognized in this process (Schwartz and Truman, 1984): (1) muscle atrophy brought about by declining ecdysteroid titre before eclosion, during which the muscle is greatly reduced in bulk but retains intact fibrils, nuclei and some mitochondria, and (2) complete degeneration and cell death immediately after eclosion, due to the action of eclosion hormone. In *Manduca*, both the atrophy and the final degeneration are apparently triggered by successive steps in the falling 20HE titre, and cell death is due to the activation of expression of certain new genes, including that for ubiquitin (Schwartz *et al.*, 1990). The two steps described may provide a model for understanding processes involved in the reversible and permanent degeneration, respectively, of flight muscle in other species.

3.5.4 *Modulation of specific gene expression*

3.5.4.1 *Regulation of specific gene expression by JH.* In tissues of adult insects, the production of a number of identified proteins has been shown to be modulated following withdrawal or application of JH. These may be termed 'target proteins' without, for the present, implying the nature of the molecular interactions responsible for the effect. Some JH target proteins are listed in Table 2. For most of these, genes or cDNAs have been cloned, to provide tools essential for molecular studies. The fact that most of these target proteins are secretory products of the fat body (that is, haemolymph proteins), and others are secretory products of colleterial and accessory glands, is due to the relative ease of detecting these major proteins and their transcripts. Also listed are several enzymes and proteins involved in cellular regulation, that are expressed at lower levels. It is likely that many additional lower abundance JH target proteins will be discovered when they are sought by appropriate methods.

Three types of response to JH can be distinguished: *induction*, in which a gene product is not detectable in the JH-deprived state but is actively produced in the presence of the hormone; *stimulation*, in which there is a certain level of constitutive production without JH, markedly enhanced by the hormone; and *repression*, in which JH markedly lowers production from the rate seen in its absence. Some examples of induction and repression of specific genes, assayed by their transcripts, in cockroach fat body by JH are shown in Fig. 9.

Some genes are unaffected by JH. In *Locusta*, for example, lipophorin,

TABLE 2 Some gene products regulated by JH in adult insects

Gene product	Insect	Tissue[a]	JH effect[b]	Cloned	References
Vitellogenin A,B	*Locusta, migratoria*	Fb	I	Genes	Locke *et al.*, 1987; Dhadialla *et al.*, 1987
Vitellogenin	*Leucophaea maderae*	Fb	I	–	Engelmann, 1971
Vitellogenins 1,2	*Blaberus discoidalis*	Fb	I	cDNA	Bradfield *et al.*, 1990; J. Y. Bradfield, pers. comm.
Vitellogenin	*Rhodnius prolixus*	Fb	S	cDNA	Valle *et al.*, 1993; Wang and Davey, 1993
Vitellogenin	*Athalia rosae*	Fb	I	cDNA	Hatekayama and Oishi, 1990; Kageyama *et al.*, 1994
21K protein (Jhp 21)	*Locusta migratoria*	Fb	I	cDNA, gene	Zhang *et al.*, 1993; Zhang and Wyatt, unpublished
19K protein	*Locusta migratoria*	Fb	S	cDNA, gene	Kanost *et al.*, 1988
Persistent storage protein	*Locusta migratoria*	Fb	S	cDNA	Wyatt *et al.*, 1992; Ancsin and Wyatt, 1996; J. Ancsin, J. George, W. Bendena and G. Wyatt, unpublished

Diapause protein	*Leptinotarsa decemlineata*	Fb	R	cDNA	de Kort and Koopmanschap, 1994
Aromatic hexamerin	*Blaberus discoidalis*	Fb	R	cDNA	Bradfield *et al.*, 1990; Jamroz *et al.*, 1996
Transferrin	*Blaberus discoidalis*	Fb	R	cDNA	Jamroz *et al.*, 1993
Cyanoproteins	*Riptortus clavatus*	Fb	S, R	cDNA	Chinzei *et al.*, 1992; Miura *et al.*, 1992
Ornithine decarboxylase	*Acheta domesticus*	Fb	S	–	Cayre *et al.*, 1995
Yolk proteins 1–3	*Drosophila melanogaster*	Fb, Of	S		Jowett and Postlethwait, 1980
Thymidine kinase	*Leucophaea maderae*	Of	S	–	La Pointe and Koeppe, 1984
Calmodulin	*Blattella germanica*	Of	S	–	Iyengar and Kunkel, 1995
Na^+/K^+ ATPase	*Rhodnius prolixus*	Of	S	–	Illenchuk and Davey, 1987a
Oothecin	*Periplaneta americana*	Lcg	I	Gene	Pau *et al.*, 1987
Long hyaline protein 1	*Melanoplus sanguinipes*	Ag	S		Cheeseman and Gillott, 1988b
Nicotinic receptor	*Acheta domesticus*	Ai	S	–	Stout *et al.*, 1994

Examples listed are those where JH regulation has been clearly demonstrated by characterized protein, enzyme assays, or transcript hybridization.
[a]Fb = fat body, Of = ovarian follicle, Lcg = left colleterial gland, Ag = male accessory gland, Ai = auditory interneuron
[b]I = induction, S = stimulation, R = repression (see Table 1)

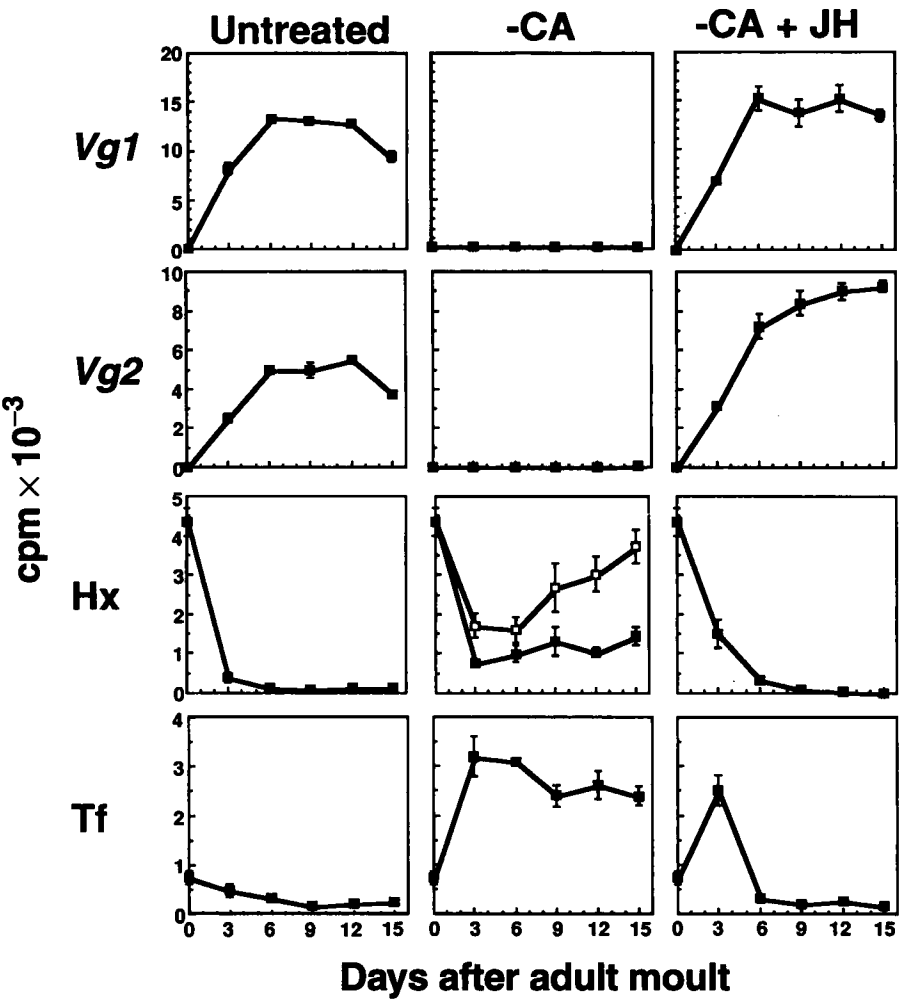


FIG. 9 Induction and repression of mRNAs by JH in fat body of *Blaberus discoidalis*. Adult female cockroaches were sampled every 3 days from the day of eclosion through the first half of the first gonotrophic cycle. One group was untreated; a second group (−CA) was allatectomized on day 0 and injected with mineral oil vector on days 2, 5, 8 and 11; a third group (−CA + JH) was allatectomized and injected on the same days with 40 μg JH III in mineral oil (n = 5–6 per group). Fat bodies were collected, total RNA was isolated, and specific transcripts were assayed by dot hybridization with ^{32}P-labelled cloned cDNAs for two *Vg* genes (*Vg1*, *Vg2*), storage hexamerin (Hx) and transferrin (Tf). mRNA levels are expressed as cpm hybridized; values are comparable for a single probe but sensitivity differed between probes. For the hexamerin, insects that were allatectomized and oil injected (−CA, ■) showed lower transcript levels that those that were simply allatectomized (−CA, □). (R.C. Jamroz and J. Y. Bradfield, unpublished data).

the haemolymph lipid-transport protein, is produced in the fat body constitutively, uninfluenced by the presence or absence of JH (Gellissen and Emmerich, 1978; Gellissen and Wyatt, 1981; Glinka and Pshennikova, 1990; Wyatt *et al.*, 1992a), and can therefore serve as a useful experimental control for comparison with JH-modulated proteins. In *Leucophaea* and *Leptinotarsa*, on the other hand, there is evidence that the haemolymph levels of the lipophorin JHBPs may be responsive to JH titre (Koeppe *et al.*, 1987; de Kort and Koopmanschap, 1992). For *Rhodnius*, the abstract in the paper of Chinzei *et al.* (1994) indicates that lipophorin is induced by JH, but this is not supported by the data presented.

For most of the examples listed in Table 2, the available data are limited to documentation of regulation by JH. The experimental study of JH regulation of gene expression has focused on the Vgs of *Leucophaea* and *Locusta* (Wyatt, 1988), and a few other fat body-expressed proteins, and the results obtained from these systems will dominate the following discussion.

The induction of Vg synthesis by JH in *Leucophaea* fat body was early shown to be completely blocked by simultaneous administration of a low dose of actinomycin D, which had relatively little effect on total protein synthesis, and from this it was inferred that *Vg* induction requires new gene transcription (Engelmann, 1971, 1972). A sensitive indicator of the presence of Vg mRNA was the formation of Vg polysomes as a sharp, fast-sedimenting peak in sucrose gradients, precipitated by Vg antibody and found only when a Ca^{2+}-dependent nuclease was inhibited by EGTA (Engelmann, 1977).

In *Locusta*, the cloning of two *Vg* genes (*VgA* and *VgB*; Locke *et al.*, 1987) provided the basis for molecular studies. The absence of detectable *Vg* transcripts in JH-deprived locust fat body and the appearance and accumulation of such transcripts after application of a JH analogue was demonstrated by translation in *Xenopus* oocytes (Chen, 1980), detection of Vg polysomes (Reid and Chen, 1981), Vg mRNA as a stainable 6.3 kb band in gels (Chinzei *et al.*, 1982) and transcript hybridization with cloned DNA probes (Dhadialla *et al.*, 1987). Recently, run-on transcription assays in isolated locust fat body nuclei have shown that *Vg* transcription rates are roughly correlated with haemolymph JH III levels during a vitellogenic cycle, and *Vg* transcription is selectively activated after application of a JH analogue to precocene-treated locusts, suggesting that JH regulates transcription of the *Vg* genes (Glinka and Wyatt, 1996). (It is of interest to note that the rate of total transcription, chiefly reflecting rRNA synthesis, also rose after JH analogue application in these experiments, but the course of total transcription during a vitellogenic cycle indicated a more complex regulation of the rRNA genes.) The possibility that JH might also enhance the survival of target gene transcripts through a JH effect on mRNA half-life, as has been shown to occur with oestradiol and vitellogenin transcripts in *Xenopus* and chicken liver (Shapiro, 1982), has not been examined. However, both for Vg in adult

Locusta fat body (Glinka and Wyatt, 1996) and for storage proteins and JH esterase in *T. ni* larval fat body (Jones *et al.*, 1993b; Venkataraman *et al.*, 1994), a lack of temporal correlation of cellular transcript levels with gene transcription rates suggests some regulation of mRNA stability.

3.5.4.2 *Stage- and sex-specificity of the JH response.* The limitation of certain genes to expression in a tissue in one sex or a particular developmental stage implies the operation of regulatory mechanisms which should be identifiable. Vg is produced in most insects only in the adult female fat body, although in some species expression begins in the last larval stage, and small amounts may be produced in males (see Section 2.1.1). Of considerable interest is the capacity for induction of Vg expression in 'unnatural' stages by high doses of JH analogues. This has been studied in several cockroaches. In *Nauphoeta cinerea* females, Vg could be induced in late larvae, and the dose of farnesyl methyl ether required declined progressively from more than 100 μg in the penultimate instar, through the final instar, to less than 1 μg in decapitated adults (Lanzrein, 1974). Similarly, in female *Blattella germanica*, progressively increasing Vg production in response to injected JH III was found from the late penultimate instar through the final instar (Kunkel, 1981). High doses of JH or analogue can induce Vg synthesis in adult males of *Diploptera punctata* (Mundall *et al.*, 1983). In *Leucophaea maderae*, Vg was not inducible in penultimate instar larvae, but was inducible in the last instar and in adults, with much higher production in females than in males (Don-Wheeler and Engelmann, 1991).

In *Locusta*, high doses of methoprene or pyriproxyfen or the implantation of active CA can induce Vg synthesis in instar V larvae of both sexes, with a much weaker response in males than in females, whereas Vg could be induced only rarely in the fourth instar and not at all in adult males (Dhadialla and Wyatt, 1983; De Kort and Koopmanschap, 1991; Edwards *et al.*, 1993; Girardie *et al.*, 1996).

There are several relevant data for proteins other than Vg. Synthesis of the hexameric persistent storage protein (PSP) of *Locusta* is repressed by experimentally applied JH analogue in the last larval instar, and stimulated by similar treatment in the adult (Wyatt *et al.*, 1992a, b; Ancsin and Wyatt, 1996). In bean bugs, *Riptortus clavatus*, cyanoprotein subunit *a* is induced in adult females and repressed in males by JH, while subunit *b* is repressed in both sexes (Section 2.1.3.4; Chinzei *et al.*, 1992a, b).

The fat body cells of a species therefore differ between stages and sexes with respect to their competence for the expression of specific genes. In several insects, the capacity for JH-induced Vg expression in females increased during the final instar to a maximum in the adult, whereas in males, the final instar showed a weak response which could either persist or disappear during the metamorphic moult. To account for the progressive increase in *Vg* gene responsiveness, one might imagine some mechanism

involving competition for a limiting essential factor. In *Locusta*, the unresponsiveness of adult male fat body is not due to lack of JH receptors, since this tissue responds to JH by stimulated DNA replication and enhanced general protein synthesis (Gellissen and Wyatt, 1981; Nair *et al.*, 1981a). In *Drosophila*, the restriction of yolk protein expression to the female is known to be controlled by the *doublesex* gene, the protein product of which occurs in two sex-specific forms, DSX^F and DSX^M, that bind to recognition elements (CTACAAAGT and CTACAAGGT) upstream from the *yp* genes and participate in the regulation of their transcription (Burtis *et al.*, 1991; Abrahamsen *et al.*, 1993; Bownes, 1994). In the DNA upstream from the *Vg* and *21K* genes of *Locusta*, similar sequence elements can be found (J. Zhang, R. Braun and G. Wyatt, unpublished), but tests of function are essential before any judgement is made as to their significance.

One feature of genes in a transcribable state in many eukaryotes is reduced methylation of cytosine at specific positions near the transcription start site (Lewin, 1994). In mammalian DNA, about 6% of the total cytosine is methylated, and altered methylation is responsible for imprinting, the differential expression of the alleles of a gene inherited from each parent. *Drosophila* DNA, however, contains little or no 5-methylcytosine (5MC), and from the few available data the content of this base in insect DNA appears to be generally very low. An early report of 5MC equivalent to 1% of the total C + 5MC in *Locusta* DNA (Wyatt, 1951) has been substantially confirmed by HPLC (1.5%; D. Swinton, S. Hattman and G. Wyatt, unpublished), and no difference was found between DNA prepared from female fat body, male fat body or testis. A search for methylation differences in the *Vg* genes of these DNA samples by use of the isoschizomer restriction enzyme technique (Lewin, 1994) revealed no differences (G. Wyatt, unpublished), but analysis of the 5′-flanking region was impeded by the presence of repetitive DNA (Locke *et al.*, 1987). An attempt to make the *Vg* genes expressible in male locusts by administration of 5′azacytidine, which cannot be methylated, was also unsuccessful (J. Bradfield and G. Wyatt, unpublished). In the light of the combined evidence, it seems unlikely that DNA methylation is important in determining the sex- and stage-related changes in transcriptional capability of genes in insects.

In other eukaryotic systems, including vertebrate animals and *Drosophila*, a prerequisite for the transcription of specific genes is the existence of an 'active state', which may reflect modified nucleosome organization, in their chromatin regions (Lewin, 1994). This can be recognized experimentally by sensitivity to deoxyribonuclease (DNAase). Gene transcription itself is often correlated with the appearance of specific localized sites of DNAase-hypersensitivity. An attempt was therefore made to investigate DNAase-sensitivity of the *Vg* genes in *Locusta* (Jensen, 1986). The technique involved DNAase and restriction enzyme treatments, followed by analysis of the DNA fragments on Southern blots, which requires the use of unique-sequence

probes from the gene regions of interest. This was frustrated by the presence of much repetitive DNA in the region upstream of the locust *VgA* and *VgB* genes (Locke *et al.*, 1987), as well as the large size of the locust genome. An indication was obtained of hypersensitive sites upstream from the transcription start site of *VgA* and 5′ of the leader exon in *VgB*, which appeared to be present only in adult female fat body, but the evidence was not entirely clear (Jensen, 1986). The question of chromatin structure in relation to JH-regulated gene expression deserves re-investigation with more advanced techniques and more favourable experimental material.

3.5.4.3 *Priming and regulation in gene induction.* Analysis of the kinetics of gene induction by JH has afforded some insight into the processes of priming and regulation that have already been described in a more general context (Section 3.3). The initiation of *Vg* expression in the fat body by JH or analogue applied to insects that have been made JH-deficient by allatectomy or precocene treatment is characterized by a remarkably long lag time. In both *Locusta* and *Leucophaea*, two days are required before accumulation of vitellogenin in the haemolymph is first detected, and in adult male *Leucophaea* the delay is extended to 4 days (Chinzei and Wyatt, 1985; Don-Wheeler and Engelmann, 1991). For the Jhp21 protein of *Locusta*, JH-induced accumulation in haemolymph is coordinate with that of vitellogenin, with a similar time-course (Zhang *et al.*, 1993). Similar kinetics are also found for the appearance of *Riptortus* cyanoprotein A in haemolymph after methoprene treatment (Chinzei *et al.*, 1982). Thus, the *de novo* appearance of several proteins after JH stimulation of JH-deprived insects involves a delay of 24 hours or more, and understanding events during this time is clearly crucial to explaining the primary actions of JH.

Studies on the induction of Vg synthesis have yielded some insight into where the delay is located. When amino acid incorporation into Vg was measured in the fat body of *Leucophaea* or *Locusta* after administration of a single dose of JH or analogue, low rates were found at 24 h, with a rapid rise thereafter and a peak at about 3 days (della-Cioppa and Engelmann, 1984b; Chen *et al.*, 1979; Edwards *et al.*, 1993). Vg mRNA in locust fat body was first detectable about 24 h after primary methoprene application (Chinzei *et al.*, 1982; Dhadialla *et al.*, 1987). Recent run-on nuclear transcription assays showed barely perceptible *Vg* transcription at 12 h after hormone application, and a steeply rising rate after 24 h (Fig. 10). Since [^{3}H]pyriproxyfen applied by the same route penetrated rapidly, attaining maximal levels in the haemolymph and fat body within 5 h (Edwards *et al.*, 1993), the delay in *Vg* gene expression must therefore largely reflect processes occurring between the arrival of the hormone analogue in its target cell and turning on transcription. Such processes fall within the category we have described as priming (Section 3.3).

The lag time can be shortened by prior exposure of the target tissue to

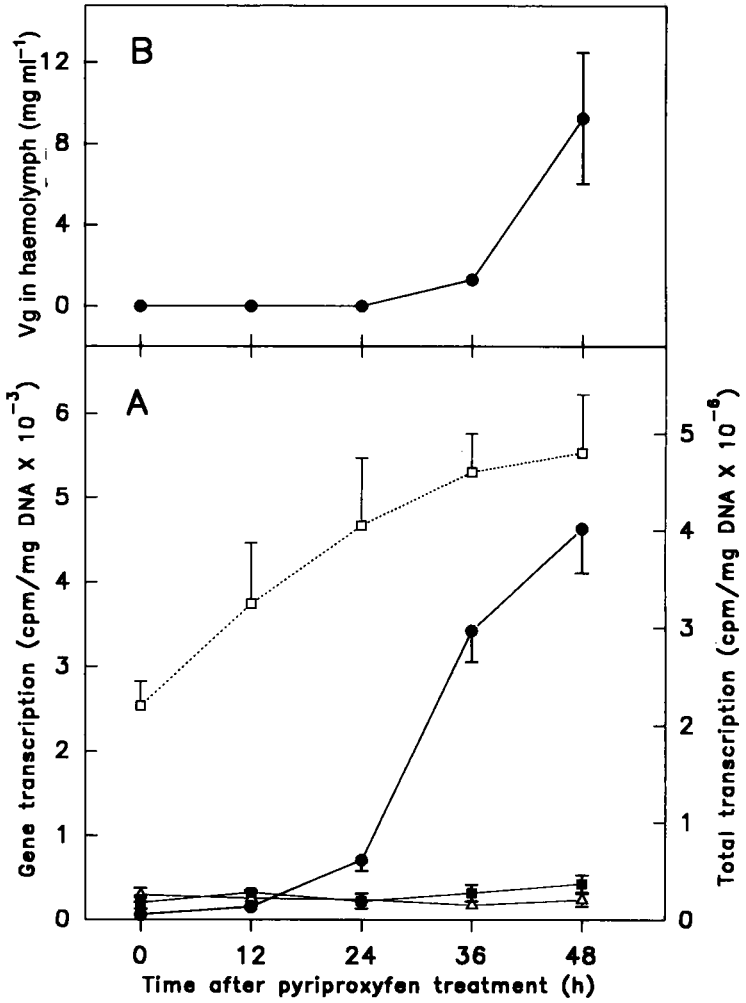


FIG. 10. Effects of a JH analogue applied to locusts on transcription in fat body nuclei. Precocene-treated adult female *L. migratoria* were treated with 10 μg of pyriproxyfen in acetone, and at intervals fat bodies were collected, nuclei were isolated and used in run-on transcription assays with [^{32}P]UTP. (A) Total transcription was determined with TCA-precipitated RNA (□), and specific gene transcription by hybridization with cloned probes for *VgB* (●), *Jhp21* (■) and *apoLpIII* (△). (B) Vg content in haemolymph sampled from the same locusts before dissection, determined by rocket immunoelectrophoresis. (Data from Glinka and Wyatt, 1996.)

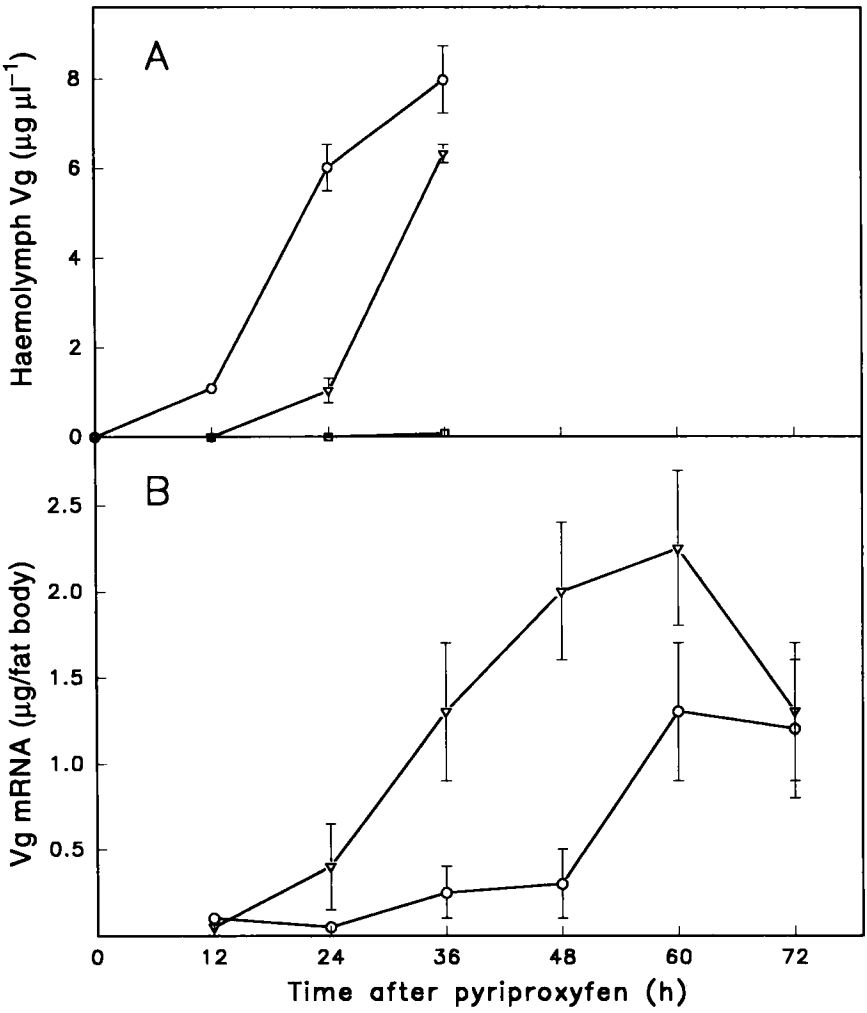


FIG. 11 Experimental alteration of the lag time for Vg induction by JH analogues in *Locusta migratoria*. All experiments were performed with precocene-treated adult female locusts. (A) The lag time is shortened by priming with a low dose of JH. Locusts were treated at 12 h intervals during 48 h with 4 doses each of 10 μg JH III in acetone (○), or with acetone alone (▽), and then (at time 0 in the figure) with 10 μg pyriproxyfen; □, 4 doses of JH III but no pyriproxyfen. After a further 12/h, 24 h and 36 h, haemolymph was sampled and its Vg content was assayed by rocket immunoelectrophoresis ($n = 4$ or 5). The priming treatment with JH III by itself induced no detectable Vg production but it accelerated the response to the inducing dose of pyroproxyfen. In other experiments, a similar priming effect was achieved

JH or an analogue. In *Blattella*, the delay for appearance of Vg in haemolymph was 16 h for last-stage larvae, but was reduced to 9 h in ootheca-bearing adults which had completed a cycle of Vg synthesis (Kunkel, 1981). In *Locusta*, secondary induction, performed by applying JH analogue to adult female locusts that had already been transiently exposed to JH or analogue, is characterized by a reduction of lag times by 12–24 hours (Chen *et al.*, 1979; Chinzei *et al.*, 1982; Chinzei and Wyatt, 1985; Dhadialla *et al.*, 1987). Recently, it has been found that an accelerated response can be quickly achieved by a low priming dose (10 μg JH III or 1 μg methoprene), given 48 h before the inducing dose (Fig. 11A). The priming dose alone failed to evoke any detectable Vg, but it brought about a more rapid response to the inducing dose. Thus, priming and regulation could be separated experimentally.

The lag phase could be lengthened, on the other hand, when protein synthesis was temporarily blocked by injection of cycloheximide at the same time as the JH analogue was applied (Fig. 11B). These results show that, in JH-deprived fat body, activation of *Vg* transcription by JH is dependent on a prior process of priming that includes the JH-induced synthesis of another protein or proteins.

The priming requirement for *Vg* induction cannot reflect a lack of tissue protein-synthesizing capacity, since the production of other proteins which are already being made can be increased without need for priming. For example, in precocene-treated locust fat body, total protein synthesis was stimulated by the wound effect of oil injection significantly earlier than Vg was induced by methoprene (Wyatt *et al.*, 1992a). For the eventual, massive production of Vg, however, build-up of the general protein-synthesizing apparatus is required, and this is seen in the proliferation of rough endoplasmic reticulum (Section 3.5.2), which is another aspect of the priming process.

It is also revealing to compare the kinetics of the processes just described with those recently reported for stimulation of JH esterase gene transcription in *Trichoplusia ni* last-instar larval fat body (Venkataraman *et al.*, 1994). In

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with a single dose of 0.6 μg of methoprene (data from Wyatt *et al.*, 1996). (B) The lag phase is extended by blocking protein synthesis. Locusts were injected with 62 μg of cycloheximide in water (○), or with water as controls (▽); one hour later they were treated with 10 μg pyriproxyfen in acetone. At intervals, groups of 3 locusts were taken for extraction of fat body RNA, and Vg mRNA was assayed by dot hybridization with a *VgB* probe; data were converted to μg of Vg mRNA by calibration with a standard RNA preparation. The cycloheximide delayed the appearance of Vg transcripts by about 24 h, which is approximately equal to the duration of inhibition of protein synthesis. (Data from Edwards *et al.*, 1993.)

this tissue, the transcription rate (from nuclear run-on assays) was elevated 3-fold within 3 h after topical application of fenoxycarb, and the tissue JH esterase mRNA content was up 6–8-fold by 12 h. These results show that JH can rapidly stimulate transcription, but the situation differs from *Vg* induction in that the JH esterase gene in the larval tissue was already active and primed for further stimulation when the hormone analogue was applied.

3.5.4.4 *Molecular events in early JH action.* Studies on steroid, retinoic acid and thyroid hormone action in vertebrate systems and ecdysteroid action in *Drosophila* have established mechanisms for the control of gene transcription that depend on binding of the hormone-receptor complex at hormone response elements, DNA sites usually located upstream from target genes (reviews: Lucas and Granner, 1992; Tsai and O'Malley, 1994; Segraves, 1994). These are sequences about 15 bp in length, containing elements of either palindromic symmetry or direct repeats, and occurring in multiple copies which function cooperatively in the regulation of a target gene. Comparisons of the DNA upstream from two locust *Vg* genes and a cockroach oothecin gene led to the identification of sequences structurally suggestive of hormone response elements (Pau *et al.*, 1987a; Wyatt, 1991). Two such sequences are associated with each of these genes, positioned between −484 and −228 from the transcription initiation sites, and they are remarkably similar in the *Vg* and oothecin genes, the only common feature of which is regulation by JH. They contain the octanucleotide AAGGGTTC, or variants thereof, which also occurs in the *Drosophila hsp27* ecdysteroid response element (Wyatt, 1991). For further consideration of these or other potential JH response elements, however, functional identification is essential – either by demonstrated activity in gene regulation or by the binding of a known JH receptor.

The electrophoretic mobility shift assay has been used for detection of protein factors involved in the activation of transcription by JH (Braun and Wyatt, 1992). Locust fat body nuclear or whole-tissue extracts were tested for proteins that could bind specifically to DNA fragments isolated from the upstream region of the *VgA* gene, and two proteins that appeared to be involved in JH action were found. JHF (JH factor), which bound to a DNA site near −600 from the transcription start site, was intensified in the transcriptionally active phase of both the normal and the pyriproxyfen-induced vitellogenic cycles. MF (mobile factor), which bound to two sites near −52 and near −600, was converted from a faster to a slower-mobility form as early as 2 h after JH analogue application, but also, more transiently, in the acetone-treated controls. It was suggested that JHF is related to JH action and is correlated with the rise in *Vg* transcription, and that MF is involved in the early steps of activation of transcription by JH and other stimuli. The factors observed may thus represent components in a cascade of gene

activation required to initiate transcription of *Vg*, but proteins specifically related to the first hours of JH action were not identified.

The DNA response elements for steroid hormones have been identified by transferring gene constructs, containing target gene promoter/enhancer sequences variously modified by *in vitro* mutagenesis, linked to a reporter gene, into hormone-responsive cells or tissues, followed by testing for hormonal modulation of expression. In attempts to use this approach for JH, constructs of *Locusta Vg* upstream DNA with CAT reporter sequences have been inserted into transgenic *Drosophila* (Wyatt *et al.*, 1986), and transferred for transient expression into *Drosophila* Kc cells (Walker *et al.*, 1991) and *Locusta* early embryos (Mathi *et al.*, 1991), but in these systems the test genes either were not expressed or were weakly expressed but failed to show stimulation by a JH analogue.

The requirements for transcription of specific genes can also be analysed in soluble nuclear extracts. This method has been successfully used with vertebrate systems such as rat liver (Gorski *et al.*, 1986) and oestrogenic regulation in chick oviduct (Bagchi *et al.*, 1992), and, in insects, for *Drosophila* embryonic gene expression (Heberlein and Tjian, 1988) and gene regulation in the silk gland of the silkworm (Hirose *et al.*, 1985; Matsuno *et al.*, 1989). Extracts prepared from locust fat body nuclei have recently been found to be active in transcription of JH regulated genes, which can be assayed by the use of reporter constructs containing a 'G-free cassette' (J. Zhang and G. Wyatt, unpublished). With protein extracts from adult female locusts, it was found that both the JH-dependent locust *Jhp21* promoter and the non-specific adenovirus major late antigen promoter (AdML) were recognized for transcription, whereas extracts from precocene-treated locust fat body transcribed strongly from the AdML but not perceptibly from the *Jhp21* promoter (Fig. 12). Precocene-treated locusts, subsequently treated for two days with pyriproxyfen, however, yielded extracts that transcribed from both promoters. The locust *VgB* gene promoter similarly supported transcription only in extracts from JH-stimulated fat body. This demonstrates that JH, applied to JH-deprived fat body, induces the synthesis of a factor (or factors) needed for the transcription of JH-dependent genes, confirming the inference from the cycloheximide experiment and priming experiments described above.

Recent experiments on transcription from the *Jhp21* promoter in locust fat body nuclear extracts have led to the identification of a DNA sequence element that strongly enhances transcription and possesses features suggestive of a hormone response element (J. Zhang and G. Wyatt, 1996 and unpublished). The sequence, AGGTTCGAG$^{A}/_{T}$CCT, which occurs at −1151 and at −2027 upstream from the *Jhp21* transcription start site, attracted attention because of its resemblance to the sequence, AGGTCAATGACCT ('IR-1'), a consensus inverted repeat which was reported to confer JH-responsiveness upon genes in mammalian cells containing FXR, a newly

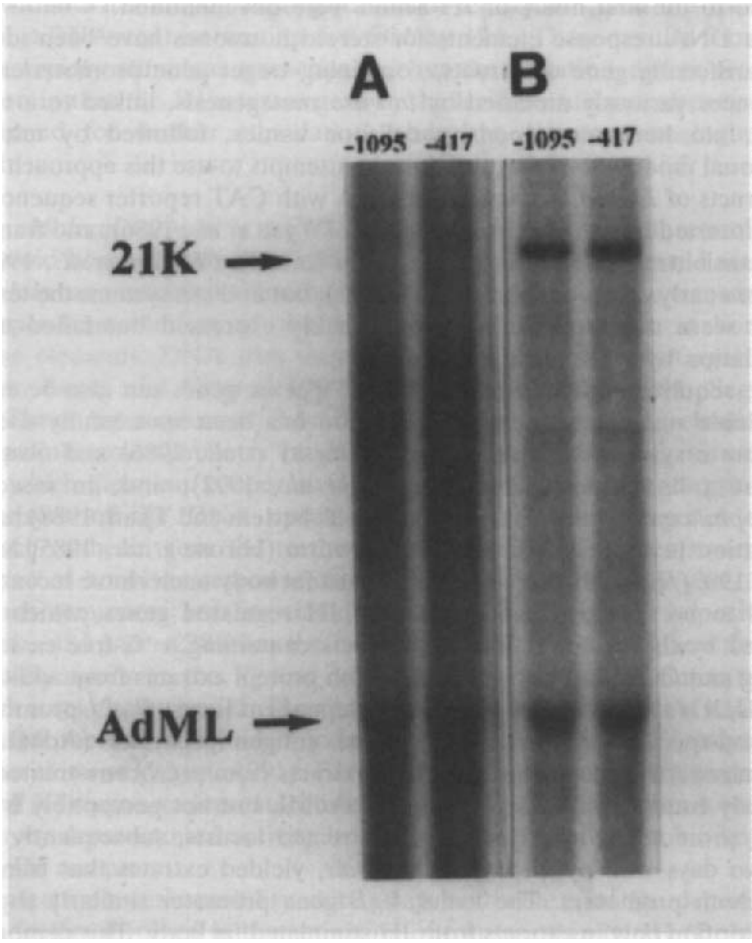

FIG. 12 Transcription from the promoter of a JH target gene in nuclear extract from *Locusta* fat body. The transcription system was prepared from adult female fat body nuclei: extract A was from locusts deprived of JH by treatment with precocene; extract B was from locusts similarly treated but administered 10 μg pyriproxyfen and dissected after 2 days. Upstream DNA (1095 nt and 417 nt) from the locust *jhp21* gene was fused to a G-free cassette of 360 bp; the control construct (AdML) contained the non-specific adenovirus major late antigen promoter fused to a G-free cassette of 180 bp. The transcription mixture included [^{32}P]UTP and transcripts were resolved by PAGE (run from the top of the figure) and visualized by autoradiography. The results show that the JH-inducible locust *jhp21* promoter supported transcription only in the extract from JH-exposed tissue, whereas the non-specific adenovirus promoter was active also in extract from JH-deprived tissue (Wyatt *et al.*, 1996).

described receptor family member (Forman *et al.*, 1995). When tested in locust nuclear extracts, the sequence between −1056 and −1200 was found strongly to stimulate transcription, and, in synthetic constructs, the 13-nucleotide element AGGTTCGAGACCT conferred equivalent enhancement. Furthermore, in electrophoretic mobility shift assays, a protein that binds to this element has been found in nuclear extracts from JH-exposed cells but not in JH-deprived cells. This evidence suggests that this sequence is a JH response element, and the protein which binds to it, a specific, JH-induced transcription factor – possibly the JH receptor itself or a protein which complexes with the JH receptor. The active DNA element bears some similarity to the sequence from *Vg* and oothecin genes, described above. The cell-free transcription system is very promising for giving further insight into the identities and functional relations of regulatory DNA elements and *trans*-acting proteins involved in gene regulation by JH.

3.5.4.5 *Polyamines.* As the major organic intracellular cations, the polyamines putrescine (1,4-diaminobutane), spermidine and spermine are important in the regulation of a number of cellular activities, particularly those involving DNA and RNA (Tabor and Tabor, 1984; Bachrach and Heimer, 1989). The enzyme ornithine decarboxylase (ODC), which catalyses the initial, rate-limiting step in polyamine biosynthesis, has an exceptionally short half-life and its activity is raised (as a result of both transcriptional and post-transcriptional events) by a wide variety of stimuli, including steroid, thyroid and polypeptide hormones, and certain hormones of plants (Russell, 1989). In the epidermis and fat body of diapausing silk moth pupae, *Hyalophora cecropia*, ODC activity and tissue polyamine levels were found to be raised by 20HE and, to a lesser degree, by control injections or wounding (Wyatt *et al.*, 1973). It was subsequently suggested that the antimetamorphic actions of JH in insects might involve inhibition of the ecdysteroid stimulation of ODC (Willis, 1981), but, in a test of this hypothesis, a polyamine peak that immediately precedes the pupal ecdysis in epidermis of *Tenebrio molitor* was found to be slightly increased, rather than repressed, by a JH analogue (Besson *et al.*, 1986).

In adult insects of several species, positive effects of JH have been found on polyamine levels and related enzyme activities. In *Drosophila*, a normal rise in ODC activity during the first 18 h after eclosion did not take place in isolated abdomens, but could be rapidly induced in them by methoprene or JHB_3 (Birnbaum and Gilbert, 1990). This activity appeared to be associated with the fat body, and the ovaries did not show elevated ODC although they also make yolk proteins. Homozygotes of the JH-deficient *apterous* mutant were non-vitellogenic and had low ODC activity; curiously, however, heterozygotes which also had low ODC were vitellogenic. Therefore, while fat body ODC activity can clearly be stimulated by JH, there does not appear to be a direct correlation with the activation of YP synthesis.

Recently, developmental and hormonal influences on polyamines in the cerebral ganglia and fat body of adult *Acheta domesticus* have been investigated (Strambi *et al.*, 1992, 1993a; Cayre *et al.*, 1993, 1995). During the first days after eclosion, polyamine levels in the fat body rose, becoming significantly higher in egg-laying females than in males or fasted or virgin females; in the nervous tissue, lower contents and smaller changes were seen. The normal increase in fat body polyamines was prevented by allatectomy, restored by injection of JH III, and raised to a lesser degree in oil-injected controls. Effects in the nervous tissue were less clear but JH did cause a significant elevation of spermidine content. Activities of ODC and S-adenosylmethionine decarboxylase (AdoMetDC, required for spermidine and spermine synthesis) rose sharply in the ganglion and fat body early in maturation of virgin females. ODC activity was strongly depressed after allatectomy and induced by JH; AdoMetDC, curiously, was higher after allatectomy but still stimulated by JH. Effects somewhat similar to those of JH on both polyamine levels and enzyme activities were evoked by ecdysone or even by injection of saline (Strambi *et al.*, 1993b). Effects were also found on phosphorylation of certain protein components *in vitro*. These results show that nervous tissue, as well as fat body, is a target for JH action, that JH has an inductive role on polyamine biosynthesis, and that spermine can modulate the phosphorylation of specific proteins.

Considering the somewhat sparse data on polyamines in insects together with the mass of information on vertebrate systems, one can suggest that early activation of biosynthetic decarboxylases and consequent elevation of tissue polyamine levels are components of the modulation of cellular activities by JH, as well as the responses to ecdysteroids and wounding. It is unlikely, however, that any key role in determining the specific effects of JH can be attributed to polyamines.

3.5.4.6 *Interactions of JH with peptide hormones.* While the insect brain is known to control vitellogenesis by regulating the production of JH in the CA, there is also a body of early experiments that indicate a direct effect of brain neurosecretion, synergistic with that of JH, on the fat body (reviewed by Davey, 1984). In several instances, the inhibitory effects of decapitation or destruction of the medial neurosecretory cells were not completely repaired by restoring JH alone, but remediation required implantation of both CA and neuroendocrine tissue from the brain or corpora cardiaca. It was then found that the storage lobe of the corpus cardiacum of *Locusta* contains a protein synthesis-stimulating factor, produced in the brain, that is rapidly released into the haemolymph after feeding and can act upon the fat body (Carlisle and Loughton, 1979, 1984; Carlisle *et al.*, 1987).

Further evidence comes from experiments with locust fat body maintained in culture *in vitro*. Despite considerable experimental effort, the effect on Vg synthesis of JH analogue added to fat body cultures was limited to the

maintenance of induction initiated *in vivo*, or to variable, low-level secondary induction *in vitro* (Wyatt *et al.*, 1987). The more optimistic data in an earlier publication (Abu-Hakima, 1981) could not be reproduced. This led to the suggestion that a synergistic factor, possibly from the brain, was missing. A direct effect of brain extract on fat body in culture was shown by Glinka and Pshennikova (1990): with fat bodies from oviposition-stage locusts, in which Vg synthesis is repressed, Vg synthesis in the presence of methoprene was stimulated about 3-fold by extract of brains from fed locusts. Brain extract from starved animals was inactive. Methoprene or brain extract alone was ineffective and the two factors acted synergistically (Glinka *et al.*, 1994b, 1995b). The brain factor of Glinka and coworkers has not been characterized, but it seems possible that it may correspond to the protein synthesis-stimulating hormone of Carlisle and Loughton (1984), or to the 6900 Da 'ovary maturating parsin' of Girardie and coworkers (Girardie *et al.*, 1991, 1992, 1996; Richard and Girardie, 1992).

A repressive influence on Vg and other protein synthesis in the fat body, however, is exerted by adipokinetic hormone (AKH), which is secreted from the corpus cardiacum into the haemolymph when the oocytes are fully grown (Moshitzky and Applebaum, 1990). Earlier experiments had shown that fat bodies taken from locusts 2 or 3 days after allatectomy, when Vg synthesis had ceased *in vivo*, resumed Vg production when incubated *in vitro*, suggesting the operation of a negative control (Pines *et al.*, 1980). Recently, it has been found that Vg synthesis can be activated in oviposition stage fat body by changes of incubation medium as a result of washing out of AKH (Glinka *et al.*, 1994b, 1995b). Since blocking transcription with α-amanitin did not prevent reactivation of Vg synthesis after the oviposition stage (Glinka and Triseleva, 1989), and since it has been found that Vg mRNA is conserved in a stored form between vitellogenic cycles (Glinka *et al.*, 1994a), it was suggested that the brain factor and AKH exert positive and negative controls, respectively, at the level of translation. Another anti-JH effect in locust reproductive maturation has been described for the brain neuropeptide neuroparsin A (Girardie *et al.*, 1987).

Possible physiological roles may be suggested for these several controls over vitellogenesis in adult female locusts (cf. Applebaum, 1983). The initiation and cyclic pattern of Vg synthesis are controlled at the transcriptional level by JH, produced cyclically by the CA (Johnson and Hill, 1975; Ferenz and Kaufner, 1981), the activity of which is influenced by population density and food intake (Dale and Tobe, 1986; Glinka *et al.*, 1994b, 1995b). Translation is subject to positive control by a brain factor as a further monitor of adequate nutrition, as well as negative control by AKH, which may inhibit vitellogenesis during oviposition and during migration, when AKH is known to be released.

A different type of synergism between JH and a neuropeptide in supporting fat body protein synthesis has been found in the cockroach, *Blaberus*

discoidalis. In decapitated adult females, prolonged application of methoprene stimulated fat body protein synthesis and ovarian development, but these remained below normal unless extracts of corpora cardiaca were also provided (Keeley *et al.*, 1988). The corpus cardiacum activity was identified as the decapeptide hypertrehalosemic hormone (HTH), and synthetic HTH was found to enhance the stimulatory effect of methoprene (Keeley *et al.*, 1991, 1994). The HTH appeared to increase the general capacity for protein synthesis through effects on the supply of metabolic energy, while the specificity of gene expression was determined by the JH analogue.

Synergistic interaction of JH with neuropeptide factors has also been noted for the male accessory gland (Section 3.4.1).

An antagonistic interaction between a neuropeptide and a membrane-level action of JH has been identified in the ovarian follicle cells of *Rhodnius*. The effects of JH on the volume of the follicle cells are antagonized by a peptide released from neurosecretory cells located in segmental abdominal neurosecretory organs (Davey and Kuster, 1980). While the peptide prevents the action of JH on the follicle cells, it does not do so by interacting with the binding site for JH I on the follicle cell membrane (Ilenchuk and Davey, 1987b). Similar protease-sensitive antigonadotropic activity against JH III effects on locust follicle cells has been identified in extracts of the thoracic ganglia in locusts (Davey *et al.*, 1993).

3.5.4.7 *A model for JH regulation of nuclear activity.* The evidence relevant to constructing a model for the action of JH in modulating gene activity in tissue of adult insects may be summarized as follows:

1. JH is a small, lipoidal molecule, which should readily cross cell membranes and has been found in nuclei after incubation with intact tissue.
2. Isolated nuclei bind JH, and JH-binding proteins can be extracted from both cytoplasmic and nuclear cell fractions.
3. JH modulates the transcription of specific genes (target genes) by induction, stimulation or repression, while other genes appear to be unaffected.
4. The responses are specific for tissue, developmental stage and sex.
5. When cells deprived of JH in the adult stage are first exposed to JH, there is a long lag time for initiation of transcription from known target genes.
6. The lag time can be shortened by prior exposure *in vivo* to JH (priming).
7. The lag time can be extended by temporary inhibition of protein synthesis.
8. In a soluble nuclear-extract transcription system, JH-inducible genes are transcribed only in extract prepared from cells previously exposed to JH.

9. In the DNA upstream of a locust JH target gene (*Jhp21*) a short, partially symmetrical sequence element has been identified that enhances transcription and binds a JH-induced nuclear protein.
10. The effects of JH are pleiotropic: in addition to modulation of identified target genes, DNA replication, production of ribosomes and intracellular membranes, and cell differentiation are promoted.

All of the above characteristics of JH action have also been recorded for one or more steroid hormones, and as a working hypothesis it seems appropriate to build a model on the basis of the mechanisms which are now known in some detail for steroids, retinoic acid and thyroid hormones. A recent review (Jones, 1995) suggests that JH workers may have been unduly blinkered by dependence on the steroid model, but a better model for the nuclear actions of JH has yet to be conceived. It is important to emphasize, however, that no evidence yet obtained proves beyond question that JH exercises a primary action within the nucleus. It has been established (Section 3.4) that JH can act directly at the cell membrane in certain tissues, and the possibility has not been eliminated – though we do not believe this likely – that the nuclear responses to JH could be transduced by an internal messenger system from primary actions at the cell membrane. A line of evidence in support of direct action of JH at the gene level which, though indirect, may be persuasive is the fact that the *Vg* genes of birds and amphibia are known to be regulated by oestradiol-receptor complex binding to linked DNA response elements (Shapiro, 1982; Slater *et al.*, 1991). The Vgs of vertebrates and invertebrates are a homologous superfamily, and it seems likely that the mechanisms used for their regulation have been conserved in principle, with a switch of hormone, through evolution.

A speculative model for JH induction of Vg synthesis, based chiefly on evidence from the fat body of *Locusta migratoria* and on concepts from steroid and thyroid hormone action, is shown in Fig. 13. Little of this scheme is established in molecular detail. JH in the haemolymph is mostly bound to a carrier protein (BP), from which it dissociates to diffuse through the plasma membrane. In the cytoplasm it reassociates with intracellular BP for transport to the nucleus and transfer to a nuclear receptor. Gene *A* is an immediate-response gene requiring only binding of the JH:R complex at an upstream response element to initiate its transcription, and encoding a hormone-specific factor (TF) that is needed for the activation of the delayed-response gene *B*. Gene *B* requires both JH and the product of gene *A*; gene *B* may differ from gene *A* by needing a higher concentration of JH:R (if the product of gene *A* is R), or a higher order complex (if, for example, the product of gene *A* is a dimerization partner for R). It is possible that TF, the product of gene A, is the JH receptor, R, itself.

Expression of gene *A* represents one component of the process we have described as priming, while the activation of gene *B* represents regulation.

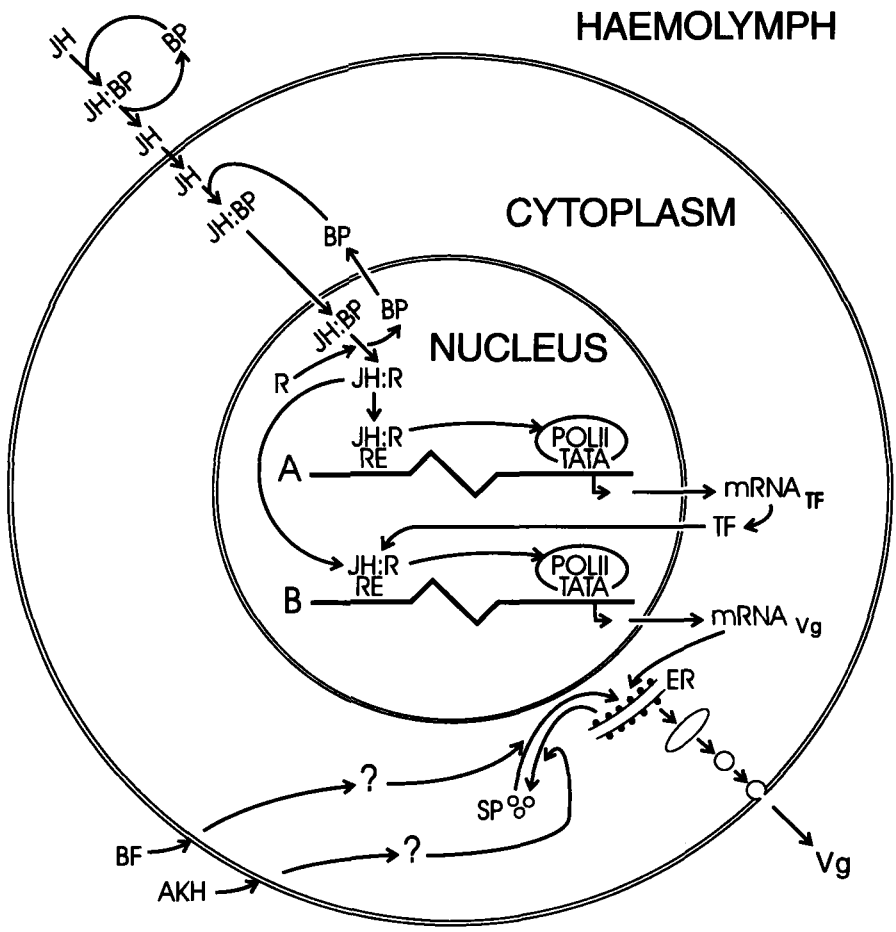

FIG. 13 A speculative model for the induction of Vg synthesis in locust fat body cells by JH, based on the available evidence for this system and on concepts taken from steroid and thyroid hormone action. JH, released from JH-binding protein (BP) in the haemolymph, diffuses through the plasma membrane and is bound by intracellular BP. The complex enters the nucleus and the JH is transferred to the nuclear receptor (R), which has a higher JH-binding affinity than does BP. The JH-receptor complex (JH:R) binds to JH response elements (RE) located in the DNA upstream from JH target genes and, by interaction with the transcription initiation complex (shown as an ellipse surrounding RNA polymerase II, POL II), activates transcription of gene *A*. Gene *A* encodes a hormone-specific transcription factor (TF) that is required for expression of gene *B*. The gene *A* transcript passes to the cytoplasm and is translated into protein (TF), which returns to the nucleus and participates, together with JH:R, in the activation of transcription of gene *B*. TF also participates in the activation of other members of a set of fat body-specific JH target genes. The gene *B* transcript passes to the cytoplasm, binds to ribosomes to form polysomes in rough endoplasmic reticulum (ER) and is translated into Vg, which

We postulate that JH:R must act on both of these genes because JH has been shown to be needed in both the priming and regulation steps of locust *Vg* expression. The proposed looping of the DNA to allow contact between JH:R and the transcription complex is based on the current model for steroid hormone action. A transcription initiation complex containing multiple protein factors, binding at the TATA box, is established for many eukaryotic systems (Lewin, 1994). Tissue-specific factors responsible for establishing tissue-, stage- and sex-specificity of transcription reflect the prior differentiation of the cell; one such factor could be the mobile factor (MF) of locust fat body described above (Section 3.5.4.4).

The *Vg* transcript, after processing to remove introns, passes to the cytoplasm and binds to ribosomes and membranes to form RER-bound polysomes. The polypeptide hormones, brain factor and AKH, act via cell surface receptors to release internal second messengers which exert positive and negative effects, respectively, on the transfer of Vg mRNA between polysomes and storage particles. In the RER, the mRNA is translated, and the primary polypeptide product is then passed to Golgi complexes for proteolytic processing, addition of prosthetic groups and secretion into the haemolymph.

This model is unquestionably a great over-simplification and is presented in order to stimulate criticism and experiments. The interaction of genes *A* and *B* represents a mechanism for selective amplification of a hormonal stimulus; this may in fact proceed through a cascade of several steps involving both activating and repressive factors. Gene *B*, designated as *Vg*, represents a class of genes encoding target proteins, as required for the pleiotropic effects of the hormone. Among the many key questions remaining to be answered by molecular genetic techniques are the nature of the JH receptor, the identities of the immediate and early genes involved, and their roles in the regulation of the late target genes.

4 Conclusions

Among animal hormones, JH is distinctive because of its unique structure and the diversity of its effects on insect development and reproduction. We

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undergoes post-translational modification in the Golgi system and is secreted into the haemolymph. The efficiency of translation is modulated by neuropeptides (brain factor, BF, and adipokinetic hormone, AKH) which, via unidentified signal transduction systems (?), shift the Vg mRNA between translationally active polysomes and inactive RNA storage particles (SP). This model is intended to provide a heuristic framework and is undoubtedly much over-simplified.

have reviewed actions of JH on the fat body, gonads, accessory glands, muscle and nervous system of adult insects. Whereas the epidermis is a major target of premetamorphic JH action (Riddiford, 1994), it has been little studied in adult insects, which generally do not moult. However, since the development of yellow pigmentation that accompanies sexual maturation in adult male locusts is clearly dependent on JH (Pener *et al.*, 1972; Pener and Lazarovici, 1979), and histological changes in the adult epidermis have been shown to follow allatectomy (Loher, 1960), the adult epidermis of at least some species must be a JH target organ.

Despite extensive literature on the biological roles of JH, it is surprising that research on its cellular and molecular modes of action has been pursued in relatively few laboratories, using only a few experimental insect systems. A large part of the present review is devoted to reporting studies describing JH-regulated processes in which cellular and molecular mechanisms remain to be investigated. We hope to stimulate research on these problems.

The progress of research on mechanisms of JH action has been delayed, and researchers may have been deterred from entry into this field, by the lack of several key experimental capabilities. The rapid recent progress in understanding how ecdysteroids regulate gene activities has resulted from the opportunities afforded by *Drosophila melanogaster*: mapped and characterized mutants, polytene chromosomes with puffs marking active genes, efficient germ-line transformation (Segraves, 1994). So far, although JH is clearly required for normal development and reproduction of *Drosophila* (Riddiford and Ashburner, 1991; Section 2.1.2), specific genes and gene products regulated by JH alone in *Drosophila* have not been identified. Recent studies on JH action in a *Drosophila* embryonic cell line, in which the ecdysterone induction of a gene is inhibited by a JH analogue (Berger *et al.*, 1992) and on the nature of the defect in the *methoprene-tolerant* mutant (Shemshedini and Wilson, 1990), are steps in the right direction.

Early events in hormone action can best be analysed when a target tissue or cells give a specific response to hormones, analogues or antagonists added *in vitro*, but such systems are now available for only certain examples of JH action. The rapid, membrane-level effects of JH can be efficiently studied in ovarian follicles and male accessory glands isolated *in vitro*, but difficulty has been encountered in the development of comparable systems for the longer-term, nuclear effects in tissues such as adult fat body (Wyatt *et al.*, 1987). This may reflect inadequate appreciation of the interactions of JH with peptide hormones or other factors that may be required for cell survival and JH response, together with the assay of gene products characterized by delayed expression. An alternative is the use of cultured cell lines, but as yet cell lines giving a specific, positive response to JH have not been described. The development of such systems will provide a better basis for molecular studies.

Especially pressing is the need for techniques for testing the effects of

experimental mutagenesis on the hormonal regulation of target genes. Attempts to transfer gene constructs into locust cells and tissues for activity assay have been frustrating (Section 3.5.4.4). The isolation of cell lines responding specifically to JH would be helpful, since cultured cells are easily transfected. The development of techniques for efficiently inserting genes into differentiated tissues for measurement of transient expression would be still more valuable, permitting identification of the requirements for stage-, tissue- and sex-limited expression, and recent success in the introduction of DNA into silk gland cells by particle bombardment (Horard *et al.*, 1994) suggests a method that may be applied to other insect organs. The recent successful transcription of JH target genes in soluble extracts from locust fat body nuclei (Section 3.5.4.4) is also an important step toward identifying the *cis*- and *trans*-acting factors that control their activity.

One of the most important general conclusions from the work described in this review is the recognition of two aspects of JH action in the tissues of adult insects. *Priming* involves the preparation of cells, through JH action, for a new or enhanced specific response to a subsequent exposure to the same or another stimulus. JH can prime cells for regulation by JH, an ecdysteroid or a neural stimulus. While the initial molecular events in priming remain to be specifically identified, the process includes protein synthesis and is complex, involving expression of multiple genes. At this stage, our concept of priming is comprehensive rather than precise, but it includes both the provision of factors essential for expression of specific genes (some previously silent), and build-up of the machinery needed for quantitatively enhanced gene expression. A model for understanding some aspects of priming by JH may be found in the action of ecdysteroids, where early genes produce factors needed for expression of late genes.

Primed JH target cells may respond to subsequent exposure to JH by *regulation* of specific activities. Here, two classes of JH action, in terms of molecular mechanism, can be distinguished. JH can act directly at the cell membrane, as in ovarian follicle cells and *Drosophila* male accessory glands. These effects are rapid, initially independent of new gene expression, and involve membrane receptors and signal transduction systems. Whether such effects of JH may occur in other target tissues is a question that needs to be examined. Regulation may also occur through modulated nuclear gene expression (induction of new genes, stimulation or repression of constitutively expressed genes), as for *Vg* and other genes in the fat body. We must here repeat that, although altered transcription in response to JH has been clearly shown, and there is much evidence to suggest that this results from primary action of the hormone within the nucleus, there is as yet no conclusive evidence proving the latter assumption.

Among the questions still to be resolved is how JH enters its target cells. Since, in locusts, the haemolymph JHBP is also found at substantial levels within the cytoplasm and nuclei of target tissues, the fat body and male

accessory gland, we have suggested that it may serve to transport the hormone and protect it from degradation and non-specific binding within the cell, as it does in the haemolymph. It is likely that JH, as a small, lipophilic molecule, traverses the cell membrane in the free form, but possible roles of JHBP in relation to JH uptake need to be examined. It must also be remembered that insects belonging to different orders use three distinct classes of haemolymph JH-binding proteins, the distributions and functions of which may not be identical.

One of the most challenging problems is the nature of the JH receptors. The locust ovarian follicle cell membrane receptor appears to have been identified and to be well on the way to characterization. Intracellular JH receptors, however, remain elusive. Although many intracellular JH-binding proteins have been advanced as potential receptors, we are unconvinced that any of the relatively abundant proteins so far described is a strong candidate. By analogy with data for steroid and thyroid hormones, as well as expectations for efficient hormonal regulation of activity of a small number of target genes, we anticipate an intracellular receptor present in only tens of thousands of copies per cell, which may require hundreds of grams of target tissue for isolation in sufficient amounts for chemical characterization. A high affinity JH-binding component of locust fat body, present in this low order of abundance, is a candidate for a nuclear receptor. Attempts to characterize the *Drosophila* ecdysteroid receptor by purification from tissue extracts, however, 'were the basis of a number of heroic but unsuccessful efforts' (Segraves, 1994), until after it had been identified through molecular genetic approaches. The prospects for direct purification of the intracellular JH receptor may be similar.

Any approach to cloning cDNA or a gene for a JH receptor without first purifying the protein involves some assumption with regard to its homology or mode of action. The simplest is to assume homology with the known nuclear receptor family and use probes or primers derived from members of that family, such as retinoic acid receptors, for screening or amplification of genomic DNA or JH target tissue cDNA. Athough this method has succeeded in discovering receptor family members in *Drosophila* (Koelle *et al.*, 1992), *Manduca* (Palli *et al.*, 1992) and *Locusta* (D. Saleh and G. Wyatt, unpublished), these have not, as yet, included JH receptors. One is left wondering whether the JH receptor has diverged so greatly in sequence that it is not found by the probes used, or whether, indeed, it does not belong to the known nuclear receptor family. Secondly, one may assume that the receptor binds to DNA response elements, identify those elements through an expression system as outlined above, and use them to select specific binding proteins from cell extracts or receptor-producing clones. We believe this approach to be promising. Thirdly, one may assume that the JH receptor, like the ecdysteroid and some other nuclear receptors, forms a heterodimer

with the *ultraspiracle* protein (Yao *et al.*, 1992) and design experiments to take advantage of this affinity. If none of these assumptions proves fruitful, there may be no alternative to protein purification.

Another persistent question is the relation of the variously structured synthetic JH analogues to JH itself. With some exceptions (see Introduction), the effects of these compounds, often at very low doses, appear to mimic true JH actions. Yet, for all of the JHBPs and putative receptors so far described, the binding affinities of the synthetic JH analogues appear to be much lower than those of the natural JHs. In part this may be an experimental artifact resulting from the propensity of the analogues for non-specific binding (Braun *et al.*, 1995). In part, the great stability and persistence *in vivo* of some of these compounds may permit them to function as JH analogues despite relatively low affinity for the JH receptor. Understanding of the relationship of structure to activity can be achieved only with the availability of *in vitro* response systems and purified receptors.

In seeking clues to mechanisms of JH action, it is important to consider other, better-known hormonal systems. We have already referred to the regulation of insect genes by 20HE, and the regulation of vertebrate Vgs by oestradiol. It should be noted that, while the nuclear actions of steroid hormones are well known, it is now clear that several steroid hormones of vertebrates can also act directly at cell membranes (Schumacher, 1990; Wehling, 1991; Orchinik *et al.*, 1992). The idea that JH can act both at the cell membrane and within the nucleus is therefore not without precedent.

Retinoic acid is especially reminiscent of JH because of its structural resemblance as an isoprene derivative and its pleiotropic effects in animal growth, differentiation and morphogenesis (Petkovich, 1992). Retinoic acid, like JH, utilizes a cellular binding protein which may convey it within the target cell (Gorry *et al.*, 1994). The vertebrate receptor for 9-*cis*-retinoic acid, RXR, is homologous with USP, the *ultraspiracle* gene product of insects, and both proteins serve as heterodimerization partners for steroid (and in vertebrates thyroid) nuclear receptors (Mangelsdorf *et al.*, 1993). It is plausible to suggest that the insect JH receptor might be a homologue of the vertebrate all-*trans* retinoic acid receptor, but experiments based on this hypothesis have not yet been productive. Although JH-like effects of retinoic acid on several insects have been reported (Nemec *et al.*, 1993), no effect could be detected from a wide range of doses tested on adult *Locusta* (R. Braun and G. Wyatt, unpublished).

In seeking parallels to JH, our attention has been drawn to thyroid hormones. In structure, thyroxine is very different from JH, but there is considerable resemblance between thyroxine and the phenoxyphenoxy carbamate, fenoxycarb (Fig. 14). Functionally, there are marked similarities. Thyroxine governs metamorphosis in amphibians, but is remarkably pleiotropic in governing many processes ranging from the maturation of the

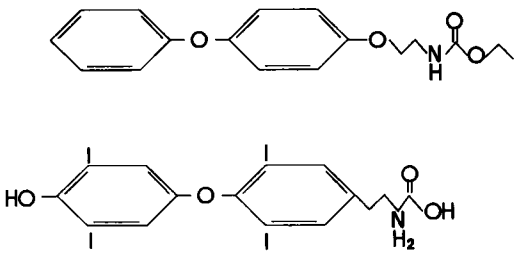

FIG. 14 Structures of the JH mimic fenoxycarb (above) and thyroxine (below).

central nervous system to thermoregulation (McNabb, 1992). Moreover, thyroxine has a variety of effects in insects (Thyagaraja *et al.*, 1993). Most of the effects of thyroxine in vertebrates can be traced to the interaction of the hormone with nuclear receptors, which belong to the steroid receptor superfamily (Chatterjee and Tata, 1992). In addition, however, thyroxine has been shown to bind to extranuclear sites that include the cell membrane, cytosolic proteins and mitochondria (Di Liegro *et al.*, 1987). The functional significance of such binding remains unclear, but some effects, such as increased membrane transport of cations or amino acids, are very rapid and independent of macromolecular synthesis (McNabb, 1992), suggesting that some of the effects of thyroxine, like those of JH, may be mediated via membrane receptors. These parallels with JH suggest that a careful examination of the models for thyroid hormone action might offer clues relevant to the actions of JH. A recent study has examined the response of ovarian follicle cells to thyroid hormones (Davey and Gordon, 1996). The follicle cells of *L. migratoria* respond to the phenoxyphenyl carbamate, fenoxycarb, *in vitro* by shrinking similarly to their response to JH, although more weakly. Thyroxine and thyronine (thyroxine lacking iodine) have a similar effect. Tri-iodothryonine (T3), the effective vertebrate hormone, however, has a more powerful effect, equal to that of JH III. The action of thyroxine, like that of JH, is inhibited by ouabain, indicating that Na^+/K^+ ATPase is involved, and by ethoxyzolamide, which is known to compete with JH for the membrane receptor. These findings may link the membrane receptor for JH III to the thyroid hormone receptor.

In suggesting guidelines for future work, we might emphasize the importance of selecting insect systems on the basis of their optimal features for the research, rather than historical precedent or economic importance. We have already drawn attention to the potential of *Drosophila*. The cockroaches, which have served well for pioneering endocrinological studies, have disadvantages for molecular work in their slow growth and reproductive development, large genome and multiple fat body cell types. The Lepidoptera that show JH regulation in the adult stage, on the other hand, have been

undeservedly neglected. With selection of appropriate systems and application of the cell and molecular research techniques now available, the elusive problem of JH action should soon yield to enlightenment.

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Physiology and Biochemistry of Insect Moulting Fluid

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1 Introduction

1.1 MOULTING FLUID, THE CUTICLE AND THE MOULT CYCLE

The periodic shedding of the insect's exoskeleton requires that a new cuticle is deposited underneath the old one, while the materials of which it is

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composed are degraded and returned to the metabolic pool prior to shedding the remains (the exuvia). While this is occurring, the space between the old and new cuticles is filled with moulting fluid. The prime function of this fluid is the degradation and recycling of the old cuticle. Moulting fluid was first described by Malpighi in his treatise of 1669 on the anatomy of the Bombycid commercial silkworm, *Bombyx mori*. Early ideas on the function of moulting fluid were vague. Rennie (1830) incorrectly supposed that its role was primarily to store and release nutrients. He correctly noted, however, that the abrupt disappearance of moulting fluid prior to ecdysis coincided with the withering and thinning of the old cuticle. The proposal that the function of the moulting fluid was the digestion of the old cuticle prior to ecdysis was first made about 90 years ago (Plotnikow, 1904; Tower, 1906).

It is now generally accepted (though this may not be exclusively true) that the moulting fluid is secreted by the epidermis. This suggests that there must exist many parallels between the cuticular epidermis and the moulting fluid enzymes on the one hand, and the gut endodermis and the digestive enzymes on the other. This idea of the moulting epidermis as an inside-out gut will recur frequently through the following pages.

Some fungal pathogens of insects gain entrance to their hosts from the body surface by attacking the cuticle with enzymes. It is perhaps not surprising that there are parallels to be drawn between these enzymes and those of the insect's moulting fluid, and we will draw attention where appropriate to this convergence of function.

1.1.1 *Cuticle structure and chemistry*

Before we can understand the role of the moulting fluid in moulting, it is necessary to know something about the cuticle that it is the moulting fluid's function to degrade.

An insect's body surface is entirely enclosed within its integumental exoskeleton, which also covers the lumenal face of the foregut, hindgut and salivary glands, and lines the trachea. The midgut endothelium does not secrete a cuticle, but it is protected by the peritrophic membrane, a chitin-containing structure that has some similarities with cuticle, but which is distinct from it (Spence, 1991). The cuticle is secreted by an epidermal cell monolayer that deposits an extracellular matrix on its outer surface. The complex layered structure of the cuticle represents a record of the temporally changing synthetic activities of the epidermal cells. A detailed review of cuticle structure and function would be inappropriate here, but there are a number of excellent accounts available to the interested reader (Neville, 1975; Hepburn, 1985; Binnington and Retnakaran, 1991). It is necessary, however, to give a brief account of these matters so that their relevance to the physiology and biochemistry of the moulting fluid may be understood (see Fig. 1).

The outermost layers of the cuticle, the cement and wax layers, are

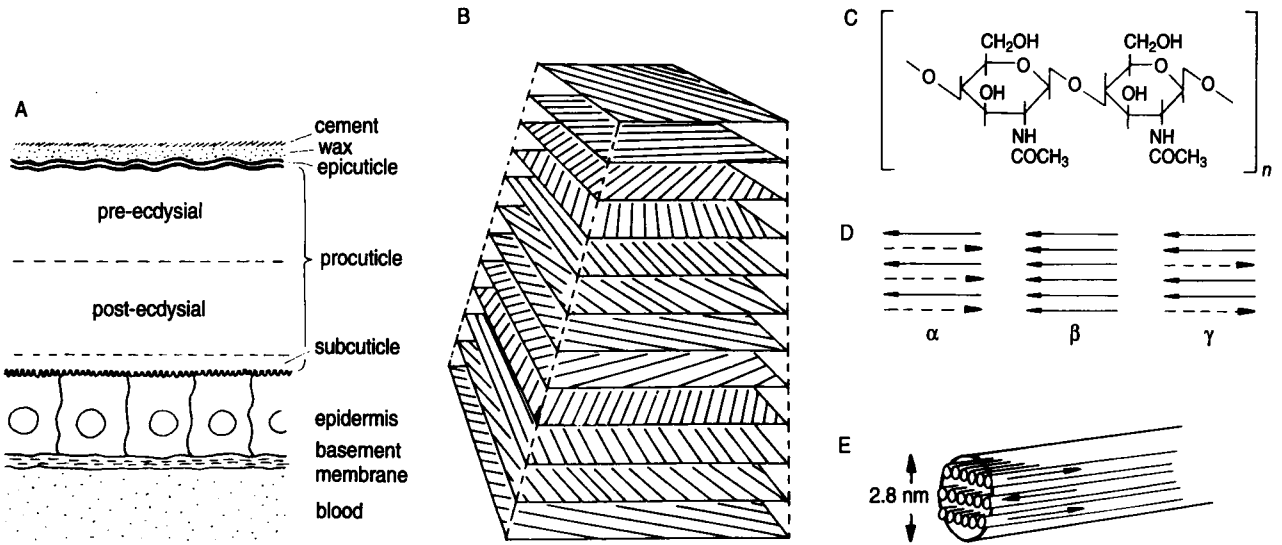


FIG. 1 The insect cuticle: (A) Highly diagrammatic representation of the layered structure of insect cuticle. (B) Helicoidal arrangement of chitin microfibrils in insect cuticle. The diagram shows how helicoidal ultrastructure leads to the illusion of parabolic patterns when the cuticle is sectioned for electron microscopy. A block of cuticle is represented as a stack of layers, each containing parallel chitin microfibrils oriented at an angle to those in the layers above and below. The direction of orientation changes in an anti-clockwise sense. Parabolae may be visualized at the edges of the block, as a result of the juxtaposition of neighbouring layers. (C) The chemical structure of chitin (poly N-acetylglucosamine). (D) Crystalline forms of chitin. Only α-chitin occurs in insect cuticle. (E) The α-chitin crystallite or microfibre, believed to contain 18 antiparallel GlcNAc polymer chains. From Reynolds, 1987.

protective in nature. They are only laid down after the rest of the cuticle has been secreted. The wax layer is secreted through pore canals in the cuticle underneath, and emerges onto the cuticle surface through fine canaliculi in the underlying epicuticle (Locke, 1960), while the cement layer is secreted by the dermal glands (modified epidermal cells) that open onto the cuticle surface (Wigglesworth, 1947; Horwath and Riddiford, 1991). The epicuticle, lying underneath the cement and wax layers, is the first part of the cuticle to be laid down during moulting (Locke, 1966). It is comprised of distinct outer and inner epicuticular layers (terminology used here is that of Weis-Fogh, 1970) and is probably mainly concerned with waterproofing. The chemical composition of the outer epicuticle is uncertain, but is probably lipidic. The inner epicuticle is believed to be protein impregnated with polyphenols (Neville, 1975). Chitin is probably absent from the epicuticle. It is less extensible than the rest of the cuticle, and it has been suggested that the size and shape of the new epicuticle effectively defines the morphology of the next instar (Bennet-Clark, 1963).

The bulk of the cuticle, or procuticle, has a primarily mechanical function, serving to confer the stiffness and strength that is required for the cuticle's function as the insect's exoskeleton. The procuticle is a fibre-composite material, composed of highly crystalline chitin microfibres embedded in a matrix constructed mainly of a mixture of structural proteins. The chitin content varies markedly depending on cuticle type and insect species. Chitin (a 1-4α polymer of N-acetylglucosamine [GlcNAc]) confers stiffness on the cuticle (Neville, 1975). Chitin can occur in nature in a number of crystalline forms (Rudall and Kenchington, 1973). The only one of these forms to occur in insect cuticle is designated α-chitin, in which neighbouring poly-GlcNAc chains run antiparallel to each other. It is thought that a single chitin crystallite contains 18 polymer chains (Neville *et al.*, 1976). The contribution of the α-chitin microfibres to the mechanical properties of the procuticle is dramatically demonstrated by the extremely deleterious consequences on the cuticle's stiffness and strength of inhibiting chitin synthesis with the acylurea insecticide, diflubenzuron (Ker, 1977). The protein component of the procuticle acts as the filler between the chitin microfibres, in which role it may confer strength on the cuticle by acting as a crack-stopper (Gordon, 1968). Many proteins are found in the cuticle matrix. These differ among insects, between regions of the body, between different types of cuticle within the insect, and also the developmental stage of the insect (Andersen *et al.*, 1995). Certain common amino acid sequence motifs recur among cuticle proteins, a point that will be discussed further in Sections 3.2 and 3.6.

1.1.2 *The moulting cycle*

The cuticle's limited capacity to expand in order to accommodate the growth in size of its owner means that the insect must periodically discard the old

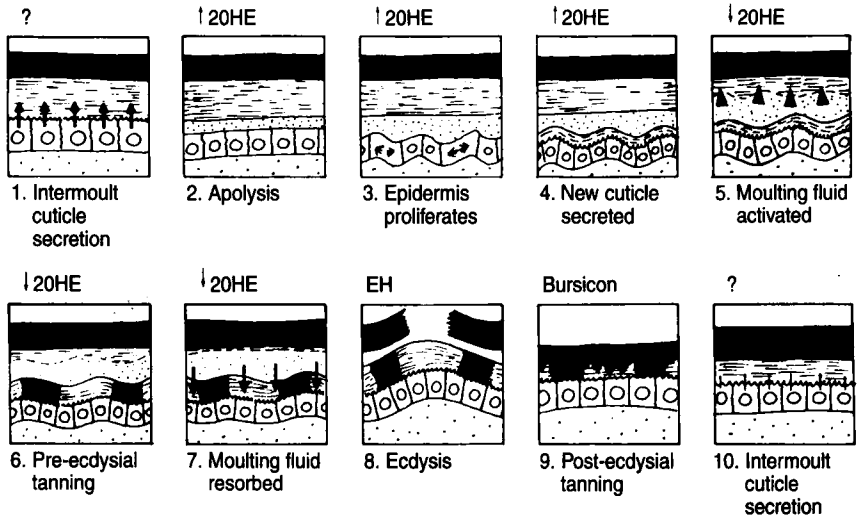


FIG. 2 The moulting cycle: Individual panels (1–10) represent cellular activities in cartoon form. The diagrams attempt to show a generalized moulting cycle. In some insects some stages may be absent. For example, soft-bodied larvae do not undergo bulk sclerotization (tanning) of the pre-ecdysial cuticle in most parts of the integument. Hormonal events thought to be responsible for integrating the cycle are indicated above each panel (hormonal control of moulting relevant to moulting fluid is discussed in Section 4). From Reynolds, 1987.

cuticle and acquire a new one. Because the insect cannot live without a cuticle, the new cuticle must be secreted beneath the old one, to be revealed only when the old one is shed. The events of the moulting cycle are illustrated schematically in Fig. 2.

Hinton was the first to point out (Hinton, 1946; Jenkin and Hinton, 1966) that this sequence of events means that the process of moulting should logically be considered to begin with the separation of the old cuticle from the underlying epidermis (apolysis). Then follows a period of the insect's life stage, or instar, in which the new cuticle is secreted under the old. Hinton referred to this period as one in which the new instar is 'pharate' (cloaked) within the one whose cuticle is visible on the outer surface. He insisted that the relevant point here is that the new cuticle, not the old one, is the one that is attached to the insect's epidermis. Thus, for example, a third-stage insect which had just begun the moulting process that leads to the moult to the fourth instar would be described as a 'pharate fourth instar'. In Hinton's scheme, the new instar actually begins with apolysis, and ecdysis, the culmination of the preparation for the moult and when the old cuticle is actually shed, should be considered as merely an event within the instar. This terminology has the disadvantage that ecdysis is much more visible than apolysis, and it is ecdysis that has traditionally been considered to mark the

beginning and end of the stages of an insect's life. Hinton's terminology has sometimes generated confusion, which is why it is explained in some detail here, but it has the great merit of underlining the importance of the events that precede ecdysis in the insect's moulting cycle.

1.1.3 *Sclerotization*

After their deposition, certain parts of the procuticle are impregnated with chemical stabilizing agents. This process is properly called sclerotization (i.e. the process that defines the sclera, or hard parts of the cuticle). It has also often been called 'tanning' by analogy with the process of leather curing. A point that is often not emphasized in accounts of sclerotization, is that it occurs both before and after the old cuticle is shed. It is true in some insects (e.g. the much studied cockroaches), that immediately after ecdysis almost all of the cuticle of the newly emerged insect is strikingly white and soft, and that this cuticle then undergoes in the hours following ecdysis a spectacular sclerotization process that involves both hardening and darkening. Because this process occurs very rapidly, and is also very obvious, much attention has been devoted to this post-ecdysial aspect of sclerotization. However, it is also true that in many insects, perhaps the majority, there are large areas of the body cuticle in which a substantial part of the procuticle is already sclerotized before ecdysis takes place. For example, in almost all insects, the mouthparts are sclerotized before ecdysis, and in adult Lepidoptera and Coleoptera, only the wings remain unsclerotized by the time the imago is ready to emerge. Moreover, the stabilization of the epicuticle occurs at quite a different time from the procuticle. According to Locke and Krishnan (1971), the outer epicuticle is stabilized by phenoloxidase action shortly after its formation, and prior to the deposition of the procuticle.

The chemistry of sclerotization is still incompletely understood despite much expenditure of research effort (Andersen, 1991; Sugumaran, 1991). It seems clear that the chemical sclerotizing agents that are deposited in the cuticle at this time are metabolites of tyrosine, and that N-acetyldopamine and β-alanyldopamine are important intermediates in their production (Hopkins and Kramer, 1991). These chemicals are not themselves sclerotizing agents, but are the substrates for various phenoloxidase enzymes (Barrett, 1991) within the cuticle. These enzymes convert N-acetyldopamine and β-alanyldopamine to reactive species which then react with sclerotizing substrates within the cuticle, acting either to cross-link cuticle proteins or to form hydrophobic polyphenolic complexes. Increased cuticle stiffness then follows because of the increased density of chemical cross-links between cuticle macromolecules, and also because of the decreased water content of the cuticle (Vincent and Hillerton, 1979). It is an important point in moulting physiology that those parts of the cuticle that have been chemically sclerotized are resistant to enzymic degradation. Thus at the next moult,

these parts of the cuticle cannot be digested, and the materials of which they are made cannot be recycled.

There is a rather confusing terminology that attempts to distinguish between those parts of the cuticle that are sclerotized and those that are not. According to this scheme, the *exocuticle* (strictly, the outer part of the procuticle) is sclerotized while the *endocuticle* (i.e. the inner part of the procuticle) is not. To make things more complicated, in some insects, there is an intermediate region, the *mesocuticle* (i.e. in between the exo- and endocuticle) which is impregnated with (presumably) lipoprotein materials, so as to change its staining properties, but which is not so heavily impregnated as the overlying exocuticle. The situation is complicated by the darkening of the cuticle. This may be a chemical process intimately connected with sclerotization, or it may be due to melanin deposition, which is not strictly connected with sclerotization, although it often happens at the same time. Melanization, for example, may occur in the endocuticle of caterpillars without sclerotization. In an attempt to simplify this matter, some authors have described the exocuticle as that part of the cuticle which is deposited before ecdysis, while post-ecdysially deposited cuticle is described as endocuticle. However, in many insects (e.g. lepidopteran caterpillars) over most of the body neither pre- nor post-ecdysially deposited cuticle will become sclerotized, while in other insects (e.g. adult locusts) sclerotization continues after ecdysis, so that both pre- and post-ecdysially, deposited cuticle will be sclerotized. We suggest that it is better to refer to 'pre-ecdysial cuticle' and 'post-ecdysial cuticle' when specifically referring to the time of deposition, and to use the term 'sclerotized cuticle' specifically of the sclerotization state of the cuticle. The terms 'exocuticle', 'mesocuticle' and 'endocuticle' are at best confusing, and should be abandoned.

These distinctions are not trivial. For example, it is essential to the success of moulting that there exist within the pre-ecdysially deposited cuticle, certain regions that are not sclerotized. These are pre-formed lines of weakness ('ecdysial lines'), where the 'exocuticle' is digested by the moulting fluid. It is in these regions that the ecdysing insect will be able to rupture the old cuticle, and subsequently escape from the exuvia (see below).

1.1.4 *The ecdysial membrane*

In many species of insect, removal of the old cuticle before ecdysis reveals a thin membrane between the old and new cuticles. This structure is not present except in the pharate insect, and is termed the ecdysial membrane. In pharate pupal *Manduca sexta* this structure is quite substantial enough to be peeled off with forceps once the overlying old cuticle has been removed (personal observation). The origin of the ecdysial membrane is reviewed in some detail by Neville (1975, pp. 31–33). At the time of deposition of the new epicuticle, the inner layers of the old cuticle (consisting as in the rest

of the procuticle of a helicoidal array of chitin microfibres in a protein matrix) become sclerotized. It is these inner layers of the procuticle that constitute the ecdysial membrane. The formation of the ecdysial membrane by sclerotization is presumed to be the result of the production of tanning enzymes by the epidermis. Locke and Krishnan (1971), in a study of moulting in the caterpillar *Calpodes ethlius*, suggested that these enzymes were produced primarily in order to sclerotize the new epicuticle, so that the production of the ecdysial membrane could be regarded as an incidental by-product of the tanning of the epicuticle. However, the situation is probably more complex than this. In a further paper on *C. ethlius*, Locke and Krishnan (1973) noted that the ecdysial membrane becomes perforated as a result of the deposition within it of dense vesicles which subsequently disappear. The dimensions of the holes are sufficiently substantial as to allow the free diffusion of macromolecules. This suggests that the ecdysial membrane does not pose a physical barrier to the resorption of the products of cuticle digestion. It is also evident from this that the ecdysial membrane does not protect the new cuticle beneath it from digestion by the enzymes of the moulting fluid. Neville (1975) notes the analogy between the ecdysial membrane and the peritrophic membrane of the gut (for an account of the latter see Spence, 1991).

Not all insects produce an ecdysial membrane. Filshie (1970) and Zacharuk (1972) say that some moulting flies and beetles, respectively, do not have any structure resembling an ecdysial membrane. Thus if the ecdysial membrane's function is a protective one, it is not essential.

1.1.5 *Cuticle degradation and recycling*

It is necessary for two reasons that the old cuticle is degraded before ecdysis can occur. First, the old cuticle must be split to allow it to be sloughed off. This is achieved by the general thinning of those parts of the old cuticle that have not been chemically stabilized. This general reduction in thickness reveals pre-formed lines of weakness ('ecdysial lines') in the sclerotized cuticle. These are regions of the cuticle in which sclerotization does not occur (see above). As degradation proceeds, the cuticle in these regions is removed until only the epicuticle remains to hold the cuticle intact. As ecdysis takes place, these parts of the cuticle can be split under the moderate tension generated by air swallowing, water swallowing, or peristaltic pumping of the haemolymph. In general, ecdysial lines are present on the dorsal thorax and at the base of the head capsule. Once these moulting lines are ruptured during the performance of ecdysis behaviour (Reynolds, 1980), the insect escapes from the exuvia by wriggling through the aperture thus created.

A second reason for degrading the cuticle before ecdysis is that the materials of which the old cuticle is made are valuable to the insect. Both protein and chitin contain nitrogen, the supply of which is often limiting to

growth. Pre-ecdysial solubilization and retrieval of the old cuticle allow the recycling of these nutrients. Both amino acids and sugars are recycled. Passoneau and Williams (1953) showed that when [^{14}C]-glycine was injected into the moulting fluid of pharate adult *Hyalophora cecropia*, the radiolabel could be recovered from the proteins of the emerged adult moth. It has been shown in crayfish (Gwinn and Stevenson, 1973a), *Locusta migratoria* (Surholt, 1975), and *Drosophila melanogaster* (Kaznowski *et al.*, 1986) that material from labelled chitin is recovered from the old cuticle and incorporated in the new cuticle. This is probably achieved by the direct reincorporation of GlcNAc, since a kinase system that phosphorylates GlcNAc directly to yield GlcNAc-1-phosphate is present in the epidermal cells (Gwinn and Stevenson, 1973b). The importance of this recycled route of chitin synthesis is underlined by the frequent observation (e.g. Fristrom, 1968; Gwinn and Stevenson, 1973a; Christiansen *et al.*, 1984; unpublished observations on *M. sexta* in our own laboratory) that labelled GlcNAc is in fact incorporated by isolated epidermis more efficiently into chitin than is any other sugar, despite the fact that GlcNAc does not appear as a precursor of chitin synthesis in the classical scheme of Candy and Kilby (1962).

2 Moulting fluid

2.1 COMPOSITION OF MOULTING FLUID

2.1.1 *Inorganic ions*

The ionic content and osmotic pressure of moulting fluid differ markedly from those of the haemolymph, so that its composition and formation are topics of some interest. The most detailed work remains that of Jungreis, who reviewed his own published and unpublished work on the moulting fluid of the pharate pupae of *Hyalophora cecropia* and *Manduca sexta* in some detail in a previous contribution to *Advances in Insect Physiology* (Jungreis, 1979). That review lists the original publications, of which we will draw attention to Jungreis (1973; 1974; 1978); Jungreis and Tojo (1973); Jungreis and Harvey (1975); and Jungreis *et al.* (1973). Jungreis (1979) drew attention to the existence of certain differences between the composition of the moulting fluid in leaf-fed and diet-fed insects. In general these differences can be characterized as a tendency for diet-fed insects to replace HCO_3^- ions by Cl^- ions, but the differences are not sufficient to alter the sense of the summary that follows, and the matter will not be given further consideration here.

The most complete figures for the moulting fluid's content of inorganic ions are for leaf-fed *H. cecropia* (Table 1). The major cation, by far, is K^+, while the major anion is HCO_3^-. The same is true for *M. sexta*, although information for this insect is less complete (Table 2). There is a deficit of anions, if only

TABLE 1. Inorganic constituents (all in mEq l^{-1}) of moulting fluid and haemolymph from pharate pupal silkmoth, *H. cecropia*

	Moulting fluid	Haemolymph
K^+	176.0 ± 8.7 (3)	52.7 ± 6.6 (3)
Na^+	1.5 ± 1.5 (2)	6.0 ± 0 (2)
Ca^{2+}	14.1 ± 0.8 (8)	13.3 ± 0.7 (8)
Mg^{2+}	15.5 ± 1.7 (6)	54.4 ± 5.6 (6)
Cl^-	24.8 ± 3.2 (14)	19.1 ± 1.7 (14)
HCO_3^-	85.7 ± 15.0 (7)	28.2 ± 6.13 (7)
Osmotic pressure (mosm l^{-1})	463.8 ± 13.7 (6)	330.5 ± 12.8 (6)

Data from Jungreis (1979).

inorganic ions are taken into account, and presumably the difference is made up of organic anions, most of which are amino acids and phosphates, although the protein concentration is quite high, and negatively charged proteins may also contribute to the electrolyte balance. Jungreis characterized the moulting fluid as 'a dilute solution of potassium bicarbonate', and pointed out that Kafatos (1968) had previously found that the labial glands (which arise as epidermal infoldings) of some saturniid silkmoths also secrete a saliva composed mostly of $KHCO_3$. The analogy is an appropriate one, since it is the profuse post-ecdysial secretion of saliva by the newly emerged adult silkmoth that activates a previously secreted proteolytic enzyme, cocoonase, which encrusts the surface of the mouthparts. This enzyme is responsible for digesting a hole in the silken cocoon that encloses the pupa, and thus enables the emerging moth to escape (Kafatos and Williams, 1964). The analogy with moulting fluid is clear. However, cocoonase is not similar to the moulting fluid proteases described so far (see Section 3.2), and saturniid saliva differs from the moulting fluid in that moulting fluid is not appreciably alkaline. Nevertheless, moulting fluid has a higher pH (pH range 7.1–7.4) than the haemolymph (pH range 6.5–6.7).

Comparison of the concentrations of ions in moulting fluid and haemolymph in both *H. cecropia* and *M. sexta* leads to the conclusion that the process of moulting fluid secretion leads to higher concentrations of K^+ and HCO_3^- ions in the moulting fluid than in the haemolymph, while Na^+, H^+ , and Mg^{2+} ions all appear in the moulting fluid at lower concentrations than in haemolymph. Cl^- ions are also present at lower concentration than in haemolymph in *M. sexta*, although this does not appear to be the case in *H. cecropia*. The concentrations of other ions are not dramatically different. Jungreis and Harvey (1975) measured transepithelial electrical

TABLE 2. Composition of moulting fluid and haemolymph from pharate pupal tobacco hornworm, *Manduca sexta*, measured 'late' in the moulting process

	Moulting fluid	Haemolymph
K^+ (mEq l^{-1})	180 ± 14	46 ± 4
Na^+ (mEq l^{-1})	100 ± 31	10 ± 4
Cl^- (mEq l^{-1})	6 ± 1	25 ± 2
pH	7.21 ± 0.07	6.54 ± 0.05
Inorganic phosphate (mM)	0.87 ± 0.24	7.30 ± 0.1
Total phosphate (mM)	47.1 ± 8.0	121.5 ± 21.1
Trehalose (mM)	0	32.0 ± 0.9
Free amino acids (mM)	41.7 ± 3.3	38.9 ± 4.1
'Polypeptides' (% w:v)	2.8 ± 0.6	1.4 ± 0.3
Protein (% w:v)	3.3 ± 0.1	1.4 ± 0.3

Data from Jungreis (1979). $n = 5$ in each case.

potentials in pharate pupae of both species, and found that the 'exuvial side' of the epithelium (i.e. the moulting fluid) was generally positive to the haemolymph (+5–10 mV in *H. cecropia*, and +15–25 mV in *M. sexta*). Since the Nernst equation would predict that the moulting fluid should be negative rather than positive to the haemolymph if K^+ ions were distributed passively across the epidermal barrier, we must conclude that K^+ ions are not passively distributed, and that an active K^+ transport process is likely to be occurring. By contrast, given the observed distribution of HCO_3^-, the Nernst relation for this ion predicts an moderate positive potential on the exuvial side of an epithelium that acts as a HCO_3^- electrode. This is what Jungreis (1979) observed, and we propose here (although Jungreis did not) that HCO_3^- ions are passively distributed. The mechanism of secretion of these ions will be considered further in Section 2.2.

2.1.2 *Organic solutes*

Jungreis's (1979) review provides values (see Table 2) for the concentrations in the moulting fluid of selected organic solutes. It may be seen that proteins, polypeptides, and amino acids are present in considerable quantity, while the storage and transport sugar, trehalose, is completely absent from the moulting fluid. This indicates that the moulting fluid is indeed a distinct compartment, separate from the haemolymph. The presence of a considerable quantity of organic phosphates (identity not determined) is interesting, particularly in the light of the presence of phosphatases in the moulting fluid (Section 3.3), although the significance of this is not clear. Tyrosine is likely to be among the amino acids found in moulting fluid of pharate pupal *M. sexta*, since the action of tyrosinase enzymes quickly makes the moulting fluid of this insect go black once it has been collected.

The function of the moulting fluid as the agent of the old cuticle's dissolution suggest that the sugars and amino acids found in it are likely to arise as products of the cuticle's digestion. There would be no trehalose in the moulting fluid because this disaccharide is not a product of chitinolysis.

2.2 SECRETION OF MOULTING FLUID

2.2.1 Enzymes

The present presumption that the moulting fluid is secreted by the epidermis has not always been the case. The origin of the moulting fluid was a topic of some interest in the first half of this century. Verson (1902) and Tower (1902; 1906) suggested that moulting fluid was secreted by the dermal glands (first described in *Bombyx mori* by Verson, 1890, and now termed Verson's glands in Lepidoptera). The attraction of this idea is clear. Because the epicuticle-lined ducts of the dermal glands lead through the new cuticle to the moulting space, the difficulty of how to transport cuticle-degrading enzymes through the new cuticle without attacking it is avoided. However, the observation by von Buddenbrock (1929) that these glands emptied only after the moulting fluid was formed, led later workers to abandon this idea. Wigglesworth (1947) showed that the dermal glands in *Rhodnius prolixus* produce the cement layer, discharging their contents only at the time of ecdysis. The organization, development and function of the dermal glands have been most recently investigated in *Manduca sexta* by Horwath and Riddiford (1991).

The key experiments ruling out the dermal glands as the sole source of moulting fluid were those of Kühn and Piepho (1938), who caused ligatured segments of *Ephestia kühniella* abdominal integument that did not contain Verson's glands to undergo moulting in isolation, and found that they nevertheless secreted moulting fluid; and those of Piepho (1938) who showed in *Galleria mellonella* that implanted fragments of integument that did not contain Verson's glands could still moult in time with their host, the digestion of the old cuticle in the implant proving that moulting fluid could be secreted without the presence of the dermal glands. The presumption is that the moulting fluid is a product of the ordinary cells of the cuticular epidermis and, as we shall see, there is good evidence that at least some moulting fluid enzymes are synthesized in this tissue, and that its cells engage in the activity of fluid transport into the moulting space. This of course still does not prove that the epidermal cells are alone the source of all of the moulting fluid, as Wigglesworth pointed out when he reviewed the matter in 1965. We may also add that the available evidence does not allow us to conclude that all regions of the epidermis participate equally in the secretion of moulting fluid.

It remains possible that other tissues contribute to the formation of the moulting fluid. Experimental evidence in support of the proposition that the gut contributes to the secretion of the moulting fluid was given long ago by Plotnikow (1904) and Shimizu (1931). In *B. mori* the surface of the newly ecdysed insect is covered with crystals of urates and oxalates which apparently originate in the Malpighian tubules. That these materials are transported from there to the moulting fluid by way of the anus was demonstrated by tying a ligature around the hind end of the insect, following which the crystals did not appear on the cuticle. However, it should be noted that oxalates and urates are normal constituents of the cuticle in many insects (in *M. sexta* larvae a prominent layer of crystals can be seen in the procuticle close to the epidermal cell surface – unpublished observations), and it is possible that the materials observed in the two papers on *B. mori* did not in fact originate in the Malpighian tubules.

The possible involvement of the gut in secreting moulting fluid was reportedly addressed experimentally by F. C. Kafatos in the 1960s using pharate adults of the saturniid silkmoth *Antheraea polyphemus*. A paper by Katzenellenbogen and Kafatos (1970) quotes the latter's unpublished observation that 'molting fluid elaboration and cuticular breakdown occur even after extirpation of the midgut at the pupal stage'. This result was said to prove that moulting fluid enzymes do not originate in the gut. Of course the experiment does not really prove this, only that the gut is not necessary for the formation of moulting fluid. The question of the extent to which the gut contributes, if at all, to the formation of the moulting fluid remains unresolved.

The issue has been given a new lease of life by recent studies on the molecular biology of the moulting fluid enzyme, chitinase. As anticipated, Kramer *et al.* (1993) found that chitinase genes are expressed at the time of moulting in the cuticular epithelium of the body wall. Less expected was their finding that the chitinase gene is also expressed at this time in cells of the gut endothelium (Fig. 3). Of course this does not prove that the chitinase synthesized in the gut is destined for the moulting fluid on the body surface. The moulting fluid of the gut might serve a purpose entirely within the gut (e.g. to digest the cuticle of the fore- and hind-gut linings, or to degrade the peritrophic membrane, a chitin-containing structure lining the midgut that is not strictly cuticular in nature – Spence, 1991). Alternatively, it may be hypothesized that the chitinase of the moulting fluid is secreted at least in part by the cells of the gut wall and subsequently transported to the space between the epidermis and cuticle of the body wall. As has been noted above, the space between the epidermis and the old cuticle is continuous between the surfaces of the body and the gut, so that movement of fluid between the two would not be impossible.

Dziadik-Turner *et al.* (1981) found that the principal β-N-acetyl-glucosaminidase of the pharate pupal moulting fluid of *M. sexta* (see below,

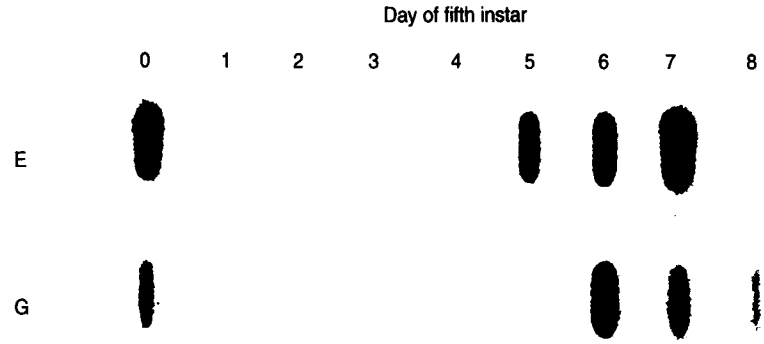


FIG. 3 Expression of moult-related chitinase in both epidermis and in gut. Slot blot analysis of *Manduca sexta* chitinase mRNAs in 2.5 μg of total RNA from day 0 through day 8 fifth instar larvae of epidermis (E) and gut (G). Numbers indicate day of fifth instar. From Kramer *et al.*, 1993.

Section 3.1.1) is also present in the insect's haemolymph. This led them to suggest that it was possible that this enzyme is synthesized elsewhere in the body, perhaps in the fat body, and transported via the haemolymph to the epidermal cells, where it would be specifically taken up and transported across the cells into the moulting fluid. This hypothesis remains untested.

A number of authors have observed that moulting fluid may contain cellular elements. Kühn and Piepho (1938) figured cells that they called 'lymphocytes' in the moulting space. Zacharuk (1972) noted that in the elaterid beetles, *Ctenicera destructor*, *Limonius californicus*, and *Hypolithus bicolor*, the moulting fluid contained isolated cells which were identified as haemocytes. The origin of these cells was not investigated, but if they were indeed haemocytes, then they must have invaded the moulting space by crossing the cuticular epidermis. These cells contained prominent lysosomes, which Zacharuk proposed contained moulting fluid enzymes which were released on the disintegration of the cells. It was suggested that at least some of the cuticle-degrading enzymic activity of the moulting fluid would thus result from the lysosomal secretions of these cells. It was pointed out that this would obviate the frequently noted difficulty that secretion of hydrolytic enzymes by the epidermal cells might be expected to damage the new cuticle that lies between the cell surface and the moulting fluid. Zacharuk suggested that only 'weaker' enzymes would be secreted by the epidermis, with 'strong' enzymes being secreted by the doomed cells stranded within the moulting space. However, recent findings on the possibly zymogenic nature of some moulting fluid enzymes, suggest to us the alternative hypothesis that the secretions of these cells may contribute to or even be entirely responsible for the activation of pre-existing proenzymes within the moulting fluid after the new cuticle has been laid down (see below).

Some commentators have pointed out that there is a difficulty in secreting active cuticle degrading enzymes through the new cuticle into the moulting fluid. If the source of the moulting fluid enzymes is indeed the cuticular epidermis, then the new cuticle is directly in the path of the newly synthesized enzymes. Richards (1951) wrote:

> 'Very suggestive also is the recent report [the then unpublished work of Passoneau and Williams] that chitinase and proteinase enzymes are to be found in the exuvial fluid of Cecropia moth pupae only for a short period during the latter part of the time when this fluid is present; sclerotization and pigmentation of the new cuticle have then already begun... In general the bulk of the procuticle is produced rather late, after the peak of molting fluid activity. Possible protection of the procuticle by the epicuticle seems inadequate as an explanation, especially if it should be found that digestive enzymes are generally not present at first. Why the newly formed or forming procuticle is not digested by enzymes that must pass out through it to reach the exuvial space remains to be explained.'

One way of avoiding this difficulty would be to secrete the enzymes in an inactive form, and to activate them only later. Where detailed studies of the time at which moulting fluid enzyme activity first appears have been done, it is evident that the enzymes are not active from the moment that moulting fluid first appears. According to Passoneau and Williams (1953), who studied the origin of the moulting fluid in the saturniid silkworm *Hyalophora cecropia*, the moulting fluid first appears at the time of apolysis. As the epidermis withdraws from the old cuticle, the exuvial space fills with 'moulting gel' containing a mixture of as yet inactive enzymes. The activation of the enzymes seems to coincide with the transport into the moulting space of additional fluid. Since Katzenellenbogen and Kafatos (1971b) found that activation of proteolytic enzymes in the moulting gel of the related *A. polyphemus* could be produced *in vitro* by artificially diluting it, it may be that activation is simply a consequence of this fluid transport into the moulting fluid. The mechanism of enzyme activation will be discussed further in Section 3.5.

2.2.2 *Fluid transport into the exuvial space*

According to Jungreis (1979), the driving force for the transfer of fluid into the moulting space is the active transport of $KHCO_3$. Section 2.1 has reviewed the evidence that these ions are considerably more concentrated in the moulting fluid of pharate pupal *H. cecropia* and *M. sexta* than in the haemolymph, and that the distribution of K^+ (but not HCO_3^-) across the epidermis is far from equilibrium. Jungreis and Harvey (1975) and Jungreis (1979) describe the results of experiments in which pieces of integument from these insects were maintained in Ussing-type chambers under voltage clamp conditions. In addition to short-circuit current measurements, in some cases

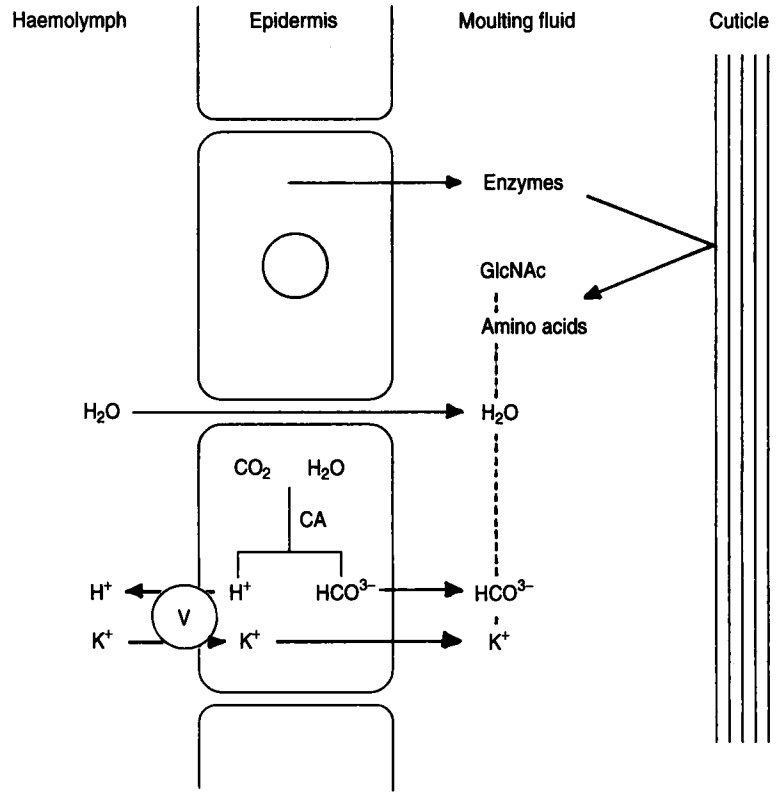


FIG. 4 Model for fluid transport across the epidermis into the moulting fluid. The model, which is based partly on the work of Jungreis (1979) on moulting fluid secretion during the lepidopteran larval–pupal moult, is highly speculative. The movement of water is osmotically coupled (dashed lines) to the transport of K^+ ions, and to the production of osmotically active cuticle digestion products. The route of water movement is unknown, but is here shown as paracellular. See text for further discussion. V = V-ATPase; CA = carbonic anhydrase; GlcNAc = N-acetylglucosamine.

K^+ fluxes were estimated by using ^{86}Rb as a tracer. These experiments will not be reviewed in detail here, and only Jungreis's conclusions will be summarized (see Fig. 4).

Briefly, Jungreis proposed that during the secretion of the moulting fluid K^+ ions are actively transported into the exuvial space by an oxygen-requiring, ouabain-insensitive process. It is likely that H^+ ions are exchanged for K^+ at the haemolymph side of the epithelial cell layer; these H^+ ions are generated within the epithelial cells by the carbonic anhydrase-catalysed splitting of CO_2. The HCO_3^- ions that are also produced by the process accompany the secreted K^+ ions into the moulting fluid. Thus the secreted

moulting fluid is more alkaline than the haemolymph, although not dramatically so (see Tables 1 and 2). Jungreis's model fits his observations quite well during the early part of the moult when short-circuit currents are essentially completely accounted for by measured K^+ fluxes, but the fit is less good closer to the time of ecdysis, when moulting fluid may be being resorbed (see Section 2.3).

The situation that Jungreis describes is strongly reminiscent of that in the much better-studied caterpillar midgut (see Dow, 1992). There, the gut endothelial cells transport K^+ ions into and H^+ ions out of the gut lumen, using a V-ATPase that has been characterized at the molecular genetic level (Dow *et al.*, 1992). However, in this case, pumping of K^+ leads to the generation of an extremely high pH in the gut lumen (up to pH 11 in *M. sexta*). We may speculate here that the reason the moulting fluid is not as alkaline as this is in part because the moulting fluid is strongly buffered by its high content of proteins, polypeptides and amino acids, phosphates, etc., and in part because the epithelium lacks the specialized goblet cells possessed by the midgut wall. Dow (1992) has explained how the specialized structure of these cells assists the gut to develop these unparalleled extreme pH values.

It is just as well that the cuticular epithelium does not produce an extremely alkaline moulting fluid, since unlike the gut enzymes which thrive at very high pH (Miller *et al.*, 1974), the moulting fluid proteases so far described do not work well at highly alkaline pH values. The pH optima for two enzymes from *M. sexta*, MFP-1 and MFP-2, are about pH 8 and pH 7 respectively (Samuels *et al.*, 1993a; b). Moreover, the ability of the epithelium to generate a transepidermal potential is rapidly and completely destroyed at pH values in excess of 10 (Jungreis, 1979).

The supposition that the entry of fluid into the moulting space is consequent upon $KHCO_3$ transport seems economical. However, it may be that once the moulting fluid enzymes begin their work, fluid entry driven by active $KHCO_3$ transport is supplemented by an additional, self-sustaining process. A point which does not seem to have been previously considered is that as the cuticle is digested, and large quantities of low-molecular-weight solutes released, the enhanced osmotic potential of the moulting fluid will necessarily draw more and more water into the exuvial space. Jungreis (1979) reported that the osmotic pressure of *H. cecropia* moulting fluid was significantly greater than that of the haemolymph (see Table 1). The high osmotic potential of this insect's moulting fluid would be expected to draw additional water into the exuvial space. Although Jungreis's figures reveal that the osmotic potential of *M. sexta* moulting fluid was not different from that of the blood, except perhaps very early during the moult, this may indicate that in this insect, differences in osmotic potential between the haemolymph and moulting fluid are transient rather than that there is a genuine species difference. Moreover, even if differences between osmotic

pressure in the two compartments are slight, this does not mean that osmotically generated water inflow does not occur. It could be that the generation of additional solutes on the exuvial side is matched rather well by osmotic water influx. Perhaps a useful way of discussing this situation is to say that unless the low-molecular-weight solutes released by moulting fluid enzymes are quickly removed from the exuvial compartment, the osmotic influx of water is inevitable. Thus, once the process of cuticle digestion has begun, at least some of the inflow of fluid into the exuvial space is likely to be self sustaining.

2.3 RESORPTION OF MOULTING FLUID

2.1.1 *Resorption across the new cuticle*

The resorption of the moulting fluid after its work of degrading the old cuticle is complete has also been seen as a problem. It has been commonly assumed (e.g. by Wigglesworth, 1965, pp. 42–43) that the moulting fluid is resorbed through the new cuticle and its underlying epidermis. In support of this proposition, Wigglesworth cited the evidence of his own paper (Wigglesworth, 1933), in which he observed in *Rhodnius prolixus* the direct resorption across the new integument of dyes which he had introduced into the moulting fluid. Others asserting that moulting fluid is resorbed across the new integument include Passoneau and Williams (1953), who watched the process occur through an experimental microscope slide window cemented to the cuticle of *H. cecropia* pupae, and Lensky *et al.* (1970), who observed the resorption of the dye buffalo black through the cuticle of the same insect. Since the moulting fluid occupies the space between the old and new cuticles, it follows that if moulting fluid is resorbed in this way, both the new cuticle and the epidermis must be permeable both to the fluids and salts of the moulting fluid and also to the products of digestion of the old cuticle. Yet the digestive enzymes themselves could not be absorbed by this route without damage to the new cuticle if they were still active during the process.

If moulting fluid is indeed resorbed through the new cuticle, this cannot have the same permeability properties as it does after ecdysis. Wigglesworth commented that the new cuticle is not yet waterproofed at the time of resorption, and that if the exuvia is peeled away then the insect will lose water through the new cuticle. This waterproofing must then be acquired later, by the secretion of lipids into the new cuticle, and perhaps by the secretion of the cement layer from the dermal glands at the time of ecdysis (see above). On the other hand, many authors (e.g. Neville, 1975) have commented that the nature of the process that digests the old cuticle but not the new, must mean that the new cuticle or perhaps its surface (the epicuticle?) must be impermeable to the moulting fluid's digestive enzymes. This paradox would be solved if the new cuticle were semi-permeable, allowing the passage of

water and small molecules (amino acids, sugars, etc.), but excluding large molecules like enzymes. The new epicuticle would thus allow only the passage of the small molecule products of cuticle digestion, and would reject the larger hydrolytic enzymes of the moulting fluid.

This situation would be rather like that in the hindgut of the intermoult insect, where the epicuticle of the cuticular intima is also semipermeable in this way, so as to allow the resorption of ions and water, and of low-molecular weight solutes such as sugars and amino acids from the gut contents prior to defaecation (Maddrell and Gardiner, 1980). However, in the case of the hindgut, the cuticle clearly possesses specialized structures to allow this recovery of materials. These take the form of epicuticular pits and depressions (Noirot and Bayon, 1969; Byers and Bond, 1971), in which the outer epicuticle's continuity is interrupted by holes, and where there are characteristic filaments within the inner epicuticle (Noirot and Noirot-Timothée, 1969). It seems likely that these pits are the sites of reabsorption of solutes and fluid from the hindgut contents. If moulting fluid resorption occurred in a similar way, then it might be expected that the newly deposited body surface cuticle would possess similar features. In fact, epicuticular pits are absent from the newly formed body cuticle. Locke (1966), however, noted that 3 nm pores could be seen in the new outer epicuticle (his cuticulin) during the pharate stage of the moulting cycle, and that these pores could conceivably act as macromolecular sieves during moulting fluid resorption. The sieving process would allow the passage of water and small solute molecules, but not of macromolecules like cuticle-degrading enzymes. This begs the question, of course, of the ultimate fate of these enzymes. Are they lost with the exuvia?

Once across the barrier of the new cuticle, there is still the epidermis to traverse. Again we meet the analogy between the cuticular epidermis and the gut. If the moulting fluid is indeed resorbed across the epidermis, the epidermal cells must possess transport mechanisms that enable the absorption of nutrients and salts, and the transport of fluids across the epidermal cell layer. However, this presumption is virtually unsupported by experimental evidence of such active transport processes. The work of Jungreis (1979) fails to shed much light on the mechanism of fluid resorption. Jungreis found that during resorption in *H. cecropia*, the transepidermal potential remains positive on the exuvial side. Radiotracer measurements in short-circuited integumental *in vitro* preparations indicated that there continued to be a net movement of K^+ ions into the moulting fluid. However, whereas during the early part of the pharate period almost all of the short-circuit current could be accounted for by the flux of K^+ ions, during the late stages of moulting (when fluid would be resorbed) the K^+ flux accounted for only about 10% of the observed short-circuit current. Jungreis concluded that other ionic species must also be crossing the cellular layer during the resorptive period. Confusingly, the presence of Ca^{2+} and Mg^{2+} caused a reduction in the

transmembrane short-circuit current compared with that seen with K^+ as the only cation, and the substitution of the only anion, Cl^-, by HCO_3^- did not alter the short-circuit current appreciably. These experiments did not allow Jungreis to advance a model for the resorption of moulting fluid in terms of active ion transport.

If moulting fluid is absorbed across the new integument, it would be necessary to transport the products of digestion of the old cuticle across the epidermis. No information at all is available on the processes of trans-epithelial amino acid and sugar transport that would be necessary to resorb the products of cuticle digestion. Here again is the analogy with the inside-out gut. It would be profitable to examine the possibility of the involvement in moulting fluid resorption of carrier-mediated nutrient uptake processes like those that operate in the cells of the insect midgut wall (see Parthasarathy *et al.*, 1994; Xie *et al.*, 1994).

One possibility is that the moulting fluid is retrieved by pinocytosis. Pinocytosis certainly occurs during the moulting process. Delachambre (1971) injected horseradish peroxidase into the ecdysial space of *Tenebrio molitor* pharate adults, and showed that the enzyme could subsequently be found in coated vesicles in the epidermis. Mauchamp and Hubert (1984) observed internalization into epithelial cells of plasma membrane glycoconjugates and plasma membrane lectins which was correlated in time with moulting fluid resorption. However, it is not clear how long this process goes on, nor how important it is to the bulk resorption of moulting fluid. It is possible that the formation of coated vesicles simply represents the recovery of membrane involved in the secretion of new cuticle materials by exocytosis (Locke, 1969), and is irrelevant to moulting fluid resorption. This is undoubtedly an area awaiting further investigation.

2.3.2 *Swallowing the moulting fluid*

Watcher (1930), working with the silkworm *B. mori*, and Zacharuk (1973; 1976), observing elaterid beetle larvae, both suggested that the retrieval of moulting fluid occurs principally by swallowing. If this is indeed the way in which the moulting fluid is removed from the moulting space, then this solves at a stroke all of the difficulties posed above of resorbing materials through the new cuticle and the epidermis. The gut is of course admirably suited for absorbing nutrient substances. There would be no need to look for transport processes in the cuticular epidermis.

Cornell and Pan (1983) reinvestigated the matter in pharate pupal *M. sexta*, and demonstrated experimentally that direct transfer of the moulting fluid to the gut occurs *via* both the mouth and the anus in this insect. Their evidence was as follows (Fig. 5). First, the disappearance of moulting fluid from the moulting space coincided with the appearance of an equivalent volume of additional fluid in the gut. Moreover, when insects were ligated

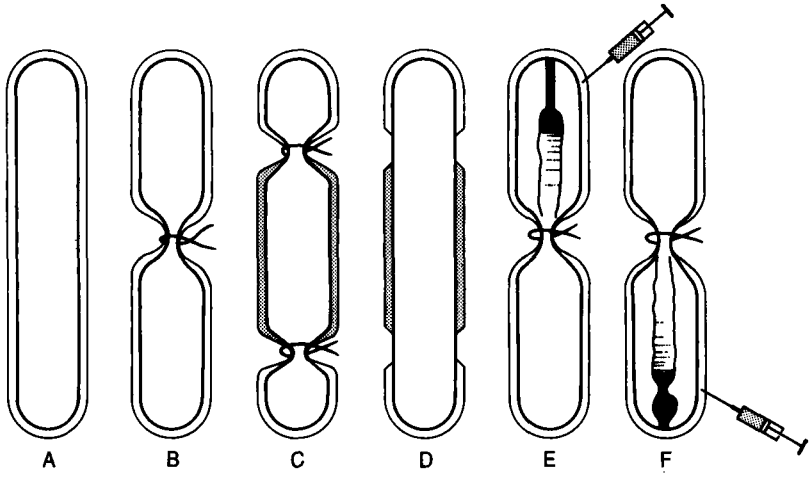


FIG. 5 Resorption of moulting fluid via the gut. Diagrammatic summary of Cornell and Pan's (1983) experiments on pharate *Manduca sexta* pupae. (A) Control. Moulting fluid is resorbed normally. (B) A ligature is tied about the middle of the pharate pupa, but fails to prevent the resorption of moulting fluid from either anterior or posterior compartments of the exuvial space. (C) A pair of ligatures isolating the central exuvial space compartment from both mouth and anus prevents resorption of moulting fluid from that compartment, but does not affect resorption from anterior and posterior compartments. (D) The same result as in (C) is seen when rings of old larval cuticle are removed and the gap sealed with superglue. This treatment does not interrupt haemolymph transport of hormones etc. (E) When methylene blue is injected into the exuvial space at the anterior end of the insect, the dye enters the foregut, and subsequently the midgut. (F) When injected at the posterior end of the exuvial space, methylene blue also enters the hindgut, and subsequently the

so that the middle of the body was isolated from both the mouth and the anus, the moulting fluid was not resorbed in this region. Single ligations failed to prevent moulting fluid resorption from moulting space compartments on either side of the ligature, so that both the front and rear openings of the gut must be capable of taking in moulting fluid. The ligatures did not exert their effects by interrupting circulation within the insect, because removal of two rings of cuticle (sealing the gap with cyanoacrylate adhesive) was just as effective as a pair of ligatures in preventing the resorption of moulting fluid from the exuvial space in between. Finally, introduction of the dye methylene blue into the moulting fluid led to the rapid appearance of dye in the foregut and/or hindgut according to the site of injection. The authors observed anterograde peristaltic waves of contraction in the hindgut of dissected insects carrying dye forward into the midgut.

Cornell and Pan (1983) did not rule out the possibility that some moulting fluid is resorbed by transepithelial transport, but considered that if it occurred

at all, this mode of transport must be a minor component of moulting fluid resorption in pharate pupal *M. sexta*. Their paper argued that the structure and properties of the integument (as reported by Jungreis, 1979) are unsuited to the rapid transcellular fluid transport that would be required to explain the observed rate of moulting fluid resorption. On the other hand, Jungreis's observations of the changed electrical properties of the epidermis at the time of resorption (see above) were not accounted for. Cornell and Pan (1983) conceded that transintegumental transport of methylene blue and [^{14}C]-inulin does occur in *Manduca* (although too slowly, the authors asserted, to account for moulting fluid resorption). They did not rule out the possibility that reabsorption across the epidermis might occur at developmental stages other than the pharate pupal stage that they examined. In particular, they observed that absorption of moulting fluid in pharate adult moths is unlikely to involve uptake via the anus. A particular problem for the present reviewers is how to explain the resorption of moulting fluid from the extremities of the appendages. It seems to us that this is very unlikely to be accomplished by swallowing alone.

As the moulting fluid is resorbed, the solutes within it are transferred to the haemolymph. Jungreis *et al.* (1982) found that there was a marked increase in blood osmolality at about the time of larval-pupal ecdysis. The osmotic potential increased from about 300 mosmol l^{-1} a few hours before ecdysis to more than 500 mosmol l^{-1} at ecdysis, returning to the previous value within 24 h. This was accompanied by an increase in the concentration of free amino acids within the haemolymph. Jungreis *et al.* (1982) commented that this brief increase in haemolymph solute concentration might be important for resorbing the moulting fluid by osmotic means prior to ecdysis. However, this suggestion supposes that the moulting fluid's solutes are absorbed separately from, and before, its content of water. This seems to us to be unlikely. We suggest that the brief increase in haemolymph osmotic pressure at the time of ecdysis is a consequence of moulting fluid resorption, rather than its cause.

It is clear that more work on this subject is needed to resolve the question of whether transepidermal resorption of fluid and/or solutes occurs at all and, if it does, how important it is.

3 Moulting fluid enzymes

To fulfil its role in moulting, the moulting fluid must contain cuticle-degrading enzymes. This was first realized by Tower (1906), but it was Wigglesworth (1933) who first showed that moulting fluid in *Rhodnius prolixus* had proteolytic activity. Chitinolytic activity in moulting silkworms, *Bombyx mori*, was subsequently demonstrated by Hamamura and Kanehara (1940), but it was not until 1955 that Jeuniaux and Amanieu showed that the enzyme

was present in moulting fluid of these insects. We now know that moulting fluid contains a complex mixture of enzymes that includes (at least) proteinases, peptidases, chitinases, β-N-acetylglucosaminidases, phosphatases, esterases and phenoloxidases. It is probable that other enzymic activities are present in addition to these.

Katzenellenbogen and Kafatos (1970) took advantage of the then novel technique of polyacrylamide gel electrophoresis to show that the moulting fluid of pharate adult *Antheraea pernyi* contains a large number of proteins. The same is true for *Manduca sexta* pharate pupae (Brookhart and Kramer, 1990) and pharate adults (Samuels and Reynolds, 1993a,b – Fig. 6). Many of these protein bands are enzymes. Brookhart and Kramer (1990) found more than 10 zones of clearing in proteinase activity gels, using gelatine as substrate. None of these clearing zones was obviously related to a moulting fluid protein band, however, indicating that enzymes may actually be minor components of the moulting fluid. Katzenellenbogen and Kafatos (1970) calculated that the total content of trypsin-like enzymes in the moulting fluid of pharate adult *A. polyphemus* was equivalent to a maximum of about 9 μg of trypsin, or 1% of the total protein in the moulting fluid. The presence of many non-enzymic proteins in the moulting fluid ought not be surprising, since the process of cuticle digestion will inevitably give rise to a large number of soluble protein and peptide fragments.

3.1 CHITINOLYTIC ENZYMES

Chitin is a distinctive component of the insect cuticle and attention has long focused on the degradation of chitin during moulting. Bade and Wyatt (1962) showed that the chitin content of *H. cecropia* cuticle is reduced by approximately 90% during the moult, indicating the presence of a highly active chitinolytic system.

Enzymic hydrolysis of cuticular chitin is a complex process (Kramer *et al.*, 1985; Kramer and Koga, 1986). In all cases, enzymes of more than one type are involved. Usually, there are multiple isoforms of enzymes of each type. Two types of chitinolytic enzymes have been designated. Those enzymes that cleave the GlcNAc polymer chains internally are described as chitinases (EC 3.2.1.14). Those removing GlcNAc monomers from chain ends are described as β-N-acetylglucosaminidases (EC 3.2.1.30). These enzymes act in sequence. Thus, chitin is first cleaved by endo-acting chitinases. The soluble oligosaccharide products of the actions of these enzymes are subsequently hydrolysed to GlcNAc by exo-acting β-N-acetylglucosaminidases (Kramer *et al.*, 1985; Kramer and Koga, 1986).

It is relevant to point out that although the crystalline form of microfibrillar chitin (which is always the α-form in insect cuticle – see Neville, 1975) is likely to have a strong influence on the process of enzymic chitin degradation, this fact has received rather little experimental attention. While some chitinase

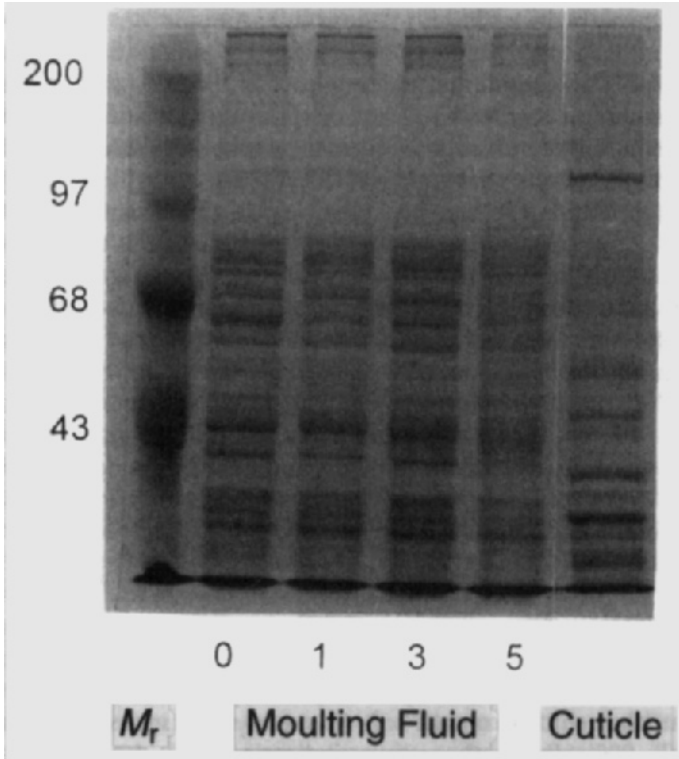


FIG. 6 SDS-polyacrylamide gel separation of proteins from pharate adult *Manduca sexta* moulting fluid and pupal cuticle. For moulting fluid (lanes 2–5), the numbers under each lane indicate the stage of the donor insect's pre-ecdysial development according to the criteria of Samuels and Reynolds (1993a – see Fig. 9 for timings). Cuticle was from abdominal segment 4 of pharate pupal cuticle (lane 6). Lane 1 is a molecular weight marker mix (M_r). Numbers at left indicate molecular weights. Unpublished data of P. Löw and S. E. Reynolds.

enzyme assays make use of native (i.e. microfibrillar) chitin, other procedures use chitin in a 'solubilized' form as colloidal or glycol chitin. Kramer *et al.* (1985) discuss in detail the merits and demerits of various ways of preparing chitin for use as a substrate in chitinase assays. It should be noted that some enzymes which almost certainly do not have any chitinolytic function in life, show activity against colloidal chitin *in vitro*. In particular, lysozyme can have detectable β-glucosaminidase activity against some preparations of colloidal chitin (Berger and Weiser, 1957), so that the presence of significant amounts of lysozyme in insects, particularly after bacterial challenge (Mulnix and Dunn, 1994), needs to be borne in mind. We have not found typical lysozyme activity (lysis of *Micrococcus lysodeikticus* cell walls) in moulting fluid from pharate adult *Manduca sexta* (S. E. Reynolds and A. M. Brown, unpublished results), but future studies of moulting fluid enzymes might well pay more attention to this point.

An early report of chitinolytic enzyme activity in the exuvia of *Bombyx mori* (Hamamura and Kanehara, 1940) was followed by more detailed studies of chitinase activity in *Hyalophora cecropia* (Passonneau and Williams, 1953), and *B. mori* (Hamamura *et al.*, 1954; Jeuniaux and Amanieu, 1955). Jeuniaux measured the release of GlcNAc from chitin by enzymes in the epidermis of *B. mori* during the moult cycle (Jeuniaux, 1961; 1963), finding that activity was only present in significant amounts in the period of preparation for ecdysis. Among early reports of chitinolytic enzyme activity in insects, Tracey and Youatt (1958) and Waterhouse *et al.* (1961) found GlcNAc-releasing activity in extracts of the termite *Coptotermes lacteus*, and in the American cockroach, *Periplaneta americana*.

Kramer *et al.* (1985) in reviewing the literature on chitin degradation, list a considerable number of papers reporting the presence of chitinolytic enzymes, and comment that in many of them it is impossible to tell whether the studies were of endo- or exo-chitinases, or both. For example, Waterhouse and McKellar (1961) reported that the highest chitinase activity was present in the integument of the cockroach, but also found activity in gut, haemolymph and saliva. It is likely that the activity reported outside the integument was really β-N-glucosaminidase, whereas that in the integument could well have measured the activity of both this enzyme and endo-acting chitinase.

Among the first studies to distinguish between endo-acting chitinase and exo-acting β-N-glucosaminidase activities in moulting insects were those of Kimura (1976) working on *B. mori*, and Spindler (1976), who used *Drosophila hydei*.

3.1.1 *The mechanism of chitinolysis*

Much more is known about the chitinolytic enzymes of the tobacco hornworm, *Manduca sexta*, than for other insects, so that this case will be reviewed in detail.

Bade (1974) showed that 'chitinase' (GlcNAc-releasing activity) could be found in the cuticle itself; although since the assay method did not properly distinguish between endo- and exo-acting enzymes, it is not clear (indeed it is most unlikely) that a single enzyme was responsible for the observed activity. Subsequently, she determined the time course of the appearance and disappearance of this activity during the moulting cycle, finding sharp peaks that just preceded ecdysis (Bade, 1975). The presence of chitin-degrading activity in the cuticle was subsequently explained as being due to the absorption of this enzyme onto cuticular chitin from the moulting fluid, following the 'exposure' of chitin by the action of moulting fluid proteases. The accumulation of chitinolytic activity in the cuticle could be prevented by the action of protease inhibitors (Bade and Stinson, 1978a,b). This point is discussed further below (Section 3.1.5).

Some of the subsequent work of Bade and Stinson on the *Manduca* chitinase system is problematic. Bade and Stinson (1981a) found that pharate pupal *M. sexta* moulting fluid contained chitinolytic activity that was fastidious in its requirements for appropriately prepared chitin substrate and required the presence of calcium ions. They pointed out themselves (Bade and Stinson, 1981b) that a combination of both endo- and exo-acting enzymes would be required to produce this result, and that therefore the 'chitinase' they studied may in fact have comprised a complex of several enzymes. At all events, the chitin-degrading activity from *Manduca* pharate pupal moulting fluid adsorbed strongly onto colloidal chitin (Bade and Stinson, 1981b), and SDS-polyacrylamide gel electrophoresis of chitin-associated proteins revealed that 3 principal protein bands of 65, 80 and 145 kDa were present. Attempts to resolubilize the enzyme were fruitless, however, so that the identities of these bands as chitinases could not be verified.

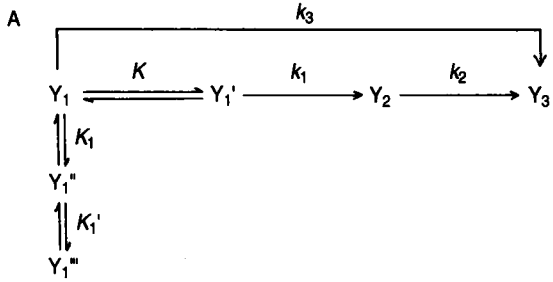
Chitinolytic activity in *Manduca* moulting fluid showed positive co-operativity in its kinetics with respect to chitin concentration (Bade and Stinson, 1979), and a model in which the enzyme displays 'processivity' (i.e. is associated with a single chitin polymer, and 'walks along' the chain) was advanced (Bade and Stinson, 1981b). The evidence in favour of this proposition was that when *Manduca* 'chitinase' is adsorbed onto colloidal chitin biosynthetically labelled with either [^{3}H]- or [^{14}C]-GlcNAc, then this label was preferentially released, even when an equal quantity of substrate labelled with the other isotope was subsequently added to the reaction mixture. This was supposed to indicate that the adsorbed enzyme could not readily transfer from one chitin chain to another. Bade and Stinson (1981c) actually recognized that there are stereochemical difficulties associated with the idea that chitinase is a 'processive' enzyme, because of the crystalline state of insect cuticular chitin. These authors have been the only researchers so far to take note of the fact that in the native microfibrillar state, neighbouring individual polymer chains of crystalline α-chitin are antiparallel. This means that if a chitinase enzyme were to show processivity, it could

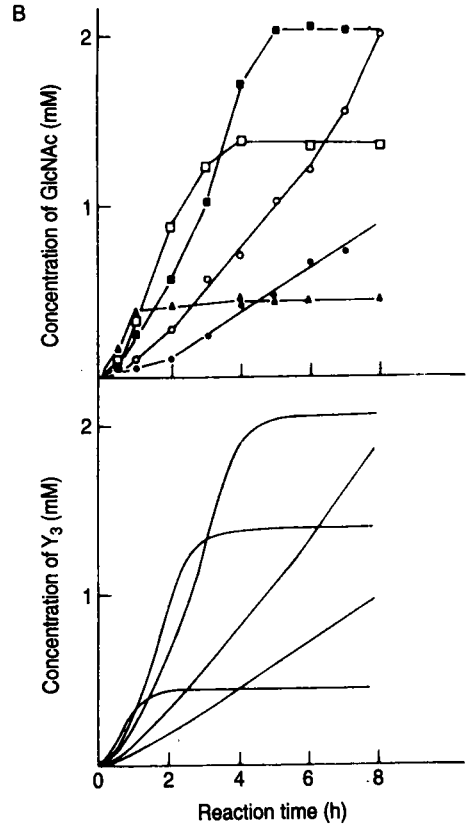
attack only those polymer chains that have the same orientation within any one microfibre (i.e. half of them). Bade and Stinson (1981c) proposed a model in which a single 'chitinase' complex has several catalytic sites which cleave chitin chains of one orientation, and are associated with one or more binding sites that fix the complex to the microfibre by binding to a polymer chain of the opposite orientation. The problem of what would happen when oppositely processing chitinase complexes met was not addressed.

These ideas have, however, been criticized by Fukamizo and Kramer (1985a; 1985b). Unlike Bade and Stinson, these workers used purified chitinase and β-N-acetylglucosaminidase (see below) and an insoluble, 'intact' chitin substrate. They measured the release of defined products (monomers and oligomers of GlcNAc) separated by HPLC. Their results were unequivocal. They found no requirement for calcium. The use of purified enzymes and intact chitin substrates abolished positive cooperativity. Chitin, whether insoluble or colloidal, was initially cleaved into oligomers, and only subsequently into monomeric GlcNAc. The larger oligosaccharide intermediates were insoluble or occurred at low levels, and the main oligomers found were N,N′,N″-triacetylchitotriose and N,N′-diacetylchitobiose. The endo-acting chitinase dominated the reaction, and combining the two chitinoloytic enzymes in various proportions showed that there was strong synergism (increasing the rate of reaction by a factor of up to six) between them. The highest synergism was obtained using a chitinase: β-N-acetylglucosaminidase ratio of 6 : 1, about the same ratio as is found in moulting fluid.

Fukamizo and Kramer's (1985a) paper explains why the binary system shows synergy when both enzymes act together. The activity of chitinase towards oligosaccharides of intermediate length (4 to 6 mers) was assayed directly using oligosaccharides prepared from crab chitin and separated by gel filtration. The course of their hydrolysis was followed by HPLC. It was found that the velocity of the hydrolytic reaction decreased markedly when the concentration of the oligosaccharide substrate was increased. This was true for 4-, 5- and 6-mer oligosaccharides, but was most marked for the shorter substrates. The inference is that the interaction between the enzyme and these oligomers is capable of multiple binding modes, some of which lead to substrate cleavage ('productive binding') and some of which lead to enzyme inhibition ('nonproductive binding'). This explains why the rapid removal of potentially inhibitory oligomers by the presence of the exo-acting β-N-acetylglucosaminidase leads to enhancement of the rate of chitinolysis by the endo-acting chitinase.

Fukamizo and Kramer (1985a) constructed a number of mathematical models of chitin hydrolysis by the binary enzyme system. The best of these models mimicked the real behaviour of chitinase with oligosaccharide substrates extremely successfully (Fig. 7). The model shown in the figure makes the following assumptions:


A
k_3
K
k_1
k_2
Y_1
Y_1'
Y_2
Y_3
K_1
Y_1''
K_1'
Y_1'''


B
Concentration of GlcNAc (mM)
2
1
Concentration of Y_3 (mM)
2
1
0
2
4
6
8
Reaction time (h)

1. a random attack mechanism with no interaction between the two enzymes;
2. monosaccharide is not produced to a significant extent from the original substrate;
3. chitinase forms both productive and nonproductive complexes with the substrate;
4. non-productive substrate binding to chitinase is more favourable than productive binding;
5. chitinase forms multiple substrate non-productive complexes; and
6. intermediate metabolite concentrations are low during the course of hydrolysis.

The success of this model leads to the conclusion (Fukamizo and Kramer, 1985a,b) that the action of chitinase is not 'processive'. Instead, the behaviour of the *Manduca* binary chitinase enzyme system *in vitro* is entirely consistent with a random model in which chitinase attacks chitin to produce oligomers which are subsequently attacked again, eventually being reduced to trimers and dimers. These then form the substrate for β-N-acetylglucosaminidase.

Thus Bade and Stinson's processive model of chitinase action is unlikely to be correct. Nevertheless, understanding how chitinase gains access to the GlcNAc polymer chains in the highly crystalline structure of the native chitin microfibre remains a problem. It should be noted that even Fukamizo and Kramer's 'intact' chitin substrate had been treated sequentially with 2M HCl, and boiling 1M NaOH, a procedure that would have left it as the deacetylated form of chitin, chitosan. This was then reacetylated by treatment with

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FIG. 7 Fukamizo and Kramer's model of the digestion of oligosaccharides by the *Manduca sexta* moulting fluid binary chitinase system. (A) Model describing the kinetics of oligosaccharide hydrolysis by a mixture of enzymes purified from pharate pupal *M. sexta* moulting fluid. Four other models were considered but were less successful in fitting observed kinetics. Y_1 = original oligosaccharide substrate; Y_2 = intermediate chitin oligosaccharide; Y_3 = N-acetylglucosamine (GlcNAc); Y_1' = productive chitinase-substrate complex; Y_1'' and Y_1''' = non-productive (dead-end) complexes for the association of one- and two- substrate molecules respectively; K = dissociation constant for Y_1'; K_1 and K_1' = dissociation constants for Y_1'' and Y_1'''; k_1 = rate constant for random cleavage by chitinase of Y_1'; k_2 = rate constant for removal of Y_3 from non-reducing end of Y_2 by β-N-acetylglucosaminidase; k_3 = rate constant for removal of Y_3 from Y_1 by β-N-acetylglucosaminidase. (B) Upper panel shows actual experimental data for the production of GlcNAc by a mixture of chitinase (32 nM) and β-N-acetylglucosaminidase (4 nM) acting on varying concentrations of an oligosaccharide substrate ($GlcNAc_6$). Lower panel shows the predictions of the model. Concentrations of substrate used in the experiments were: ● 1.52 mM; ○ 0.80 mM; ■ 0.34 mM; □ 0.23 mM; ▲ 0.07 mM. The model also successfully predicted kinetics of attack on $GlcNAc_5$ and $GlcNAc_4$. From Fukamizo and Kramer (1985a).

acetic anhydride before use. It is uncertain whether the chitin microfibres in untreated cuticle would really be as accessible to chitinase as this material.

3.1.2 *Chitinases and β-N-acetylglucosaminidases*

The work of Kramer and his collaborators on *Manduca sexta* has greatly advanced our understanding of the nature of the enzymes involved in chitin degradation. Their work shows that the 'binary' chitinase system is in fact more complex than the name would suggest.

Koga *et al.* (1983) isolated three endo-acting chitinases from *M. sexta*, all of which were present in pre-pupal integument, moulting fluid and haemolymph. Pharate pupal moulting fluid contained high levels of chitinase I (M_r 75 kDa) and smaller amounts of chitinase II (62 kDa) and chitinase III (50 kDa). Interestingly, fifth instar larval integument prior to apolysis also contained all three enzymes. Unfortunately, it was not stated whether the enzymes were present in cuticle, epidermis, or both. All three enzymes were very efficient at hydrolysing polymeric and oligomeric substrates in a typical endoenzyme cleavage pattern. So far there is no indication of any differential or specialized roles for these chitinase isozymes.

A cDNA encoding one of these *Manduca* chitinases, apparently enzyme I, was cloned and sequenced by Kramer *et al.* (1993). The nucleotide sequence encoded a 62 kDa protein consisting of 554 amino acids, although since the protein's N-terminus bore all the hallmarks of a typical signal sequence, the molecular weight of the secreted enzyme would be expected to be less than this. The inferred amino acid sequence showed strong similarity (21% identity) with chitinases from yeast, cucumber and bacteria, including two highly conserved regions that have been suggested to participate in the active site. The cloned *Manduca* chitinase sequence did not however resemble those of basic plant chitinases nor those of β-N-acetylglucosaminidases. Hybrid selection and *in vitro* translation yielded a 75 kDa protein which cross-reacted with antibodies raised against *Manduca* chitinase I. The discrepancy in molecular weight between the primary translation product and calculated size of the inferred protein sequence was thought to be due to anomalous electrophoretic migration of the enzyme. However, the situation was complicated by the fact that the native enzyme from moulting fluid was known to be glycosylated, whereas the reticulocyte translated product of the cloned gene was almost certainly not modified in this way, even though 4 consensus N-glycosylation sites were present in the sequence. Some doubt about the identity of the protein encoded by the cDNA therefore remained at the conclusion of the 1993 paper.

This was resolved by further experiments in which the enzyme was secreted *in vitro* by lepidopteran cultured cells infected with a recombinant baculovirus (Gopalakrishnan *et al.*, 1995). The *Manduca* chitinase cDNA was cloned into

the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) under the control of the polyhedrin promoter. Protein was expressed in *Spodoptera frugiperda* (SF9 and SF21) cells, and also in *Trichoplusia ni* (Hi-5) cells. The infected cells secreted an 85 kDa protein that was recognized by antibodies directed against the *Manduca* moulting fluid chitinase. In this study, the native moulting fluid chitinase I was found to have exactly the same apparent molecular size (85 kDa) as the recombinant enzyme, and to migrate similarly in native activity gels. The two proteins had the same N-terminal amino acid sequence, and were glycosylated to approximately the same extent (the native enzyme contained 24% sugars, while the recombinant protein had 27%) with similar (although not identical) proportions of the sugars glucose, N-acetylgalactosamine, N-acetylglucosamine, mannose and galactose. Thus the cDNA sequence almost certainly encodes the major chitinase (I) of the *Manduca* pharate pupal moulting fluid.

Treatment of recombinant baculovirus-infected Sf9 cells with tunicamycin (an inhibitor of glycosylation) prevented the secretion of enzyme and the production of an unsecreted protein with an apparent M_r on SDS-PAGE of 75 kDa, the same size as had been seen in the study of Kramer *et al.* (1993). N-terminal sequencing of the protein secreted into the medium by infected cells in the absence of tunicamycin revealed the same sequence (DSRARI. . .) as was present in chitinase I purified from *Manduca* moulting fluid. This is strong confirmation that the cloned cDNA indeed encodes chitinase I. It also reveals the first 19 amino acids of the protein's inferred sequence to be a signal sequence, presumably cleaved off as the protein enters the endoplasmic reticulum. The predicted molecular weight of the unglycosylated translation product after removal of the signal sequence is 60 kDa. The discrepancy between this figure and the apparent molecular weight of the protein seen in the experiment is presumably due to anomalous migration during electrophoresis.

Gopalakrishnan *et al.*'s work also revealed that the *Manduca* chitinase may be glycosylated in two stages, since although the molecular weight of the secreted enzyme was 85 kDa, an additional 80 kDa immunoreactive protein was present in baculovirus-infected cells. Since both the 80 kDa and 85 kDa proteins disappeared when the cells were treated with tunicamycin, being replaced by the 75 kDa protein, it is reasonable to conclude that both are glycosylated forms of the 75 kDa protein, and that the 80 kDa form is incompletely glycosylated. Moreover, completion of the glycosylation process must be required before secretion into the medium can occur, since the 80 kDa form of the enzyme does not occur in the medium.

β-N-Acetylglucosaminidase also occurs in *Manduca* in more than one form, although only one enzyme, EI, occurs in moulting fluid. It is also found in the integument, and in pupal haemolymph. The other enzyme, EII, occurs only in haemolymph, and it seems likely that this enzyme does not have a chitinolytic role *in vivo* (Dziadik-Turner *et al.*, 1981). EI and EII have been

purified and have the following properties: EI — M_r 61 kDa, p*I* 5.9 and pH optimum 6.0; EII — M_r 61 kDa, p*I* 5.1, pH optimum 6.0. EI is monomeric and unglycosylated, while EII is dimeric and glycosylated. The two have significantly different amino acid compositions. The two enzymes also differ in their substrate specificities. While both are active only towards β-glucosides of GlcNAc and GalNAc, EII is very much more specific than EI. Notably, EII is much less active towards the dimeric *p*-β-nitrophenyl-$GlcNAc_2$ than towards the monomeric *p*-β-nitrophenyl-GlcNAc, while EI has roughly equal activity to these two substrates. This is in accord with the idea that the function of EI in the moulting fluid is to cleave the oligomeric products of the initial attack of an endo-acting chitinase, producing monomers which are subsequently reabsorbed from the moulting fluid and utilized as precursors for synthesis of the new cuticle.

Other workers have also studied chitinolytic enzymes from various other insects. Work on another lepidopteran, *Bombyx mori*, perhaps unsurprisingly reveals a rather similar picture to that in *Manduca*. Koga *et al.* (1989) studied chitinase in the integument of *B. mori* during the larval-pupal transformation. The authors suggested that here, as in *M. sexta*, the cleavage of chitin is largely initiated by chitinases, which attack the polyGlcNAc chain in a random fashion. Active chitinase did not appear in *Bombyx* until late in the moulting cycle (*c.* one day before ecdysis) whereas active β-N-acetylglucosaminidase was present at stage SP1, one day after the spinning stage, and two days before chitinase activity was detectable. The finding that active β-N-acetylglucosaminidase fails to initiate chitin hydrolysis when it is present before chitinase, again supports the view that chitinase is the rate-limiting enzyme during chitin digestion. However, as will be seen below (section 3.1.2), there may be significant differences between the way that chitinases are activated in *Manduca* and *Bombyx*. As in *Manduca*, there is more than one form of chitinase in *Bombyx*. Kimura (1974; 1976) found that silkworm chitinase activity could be fractionated by gel filtration to give two peaks of activity. Koga *et al.* (1989) found two active forms of *Bombyx* chitinase, with M_r of 88 kDa and 65 kDa. Also as in *Manduca*, multiple β-N-acetylglucosaminidases can be found in *Bombyx* (Kimura, 1976; 1977). It is not clear how many of these occur in the moulting fluid. In extracts of whole integument, there are two forms – a homodimer consisting of 67.5 kDa subunits, and a heterodimer consisting of 57.5 and 67.5 kDa subunits (Koga *et al.*, 1989).

Thus the picture from both *Manduca* and *Bombyx* is that of multiple chitinases and β-N-acetylglucosaminidases. Things seem to be the same in other species of insect, although much less is known. An unsatisfactory feature of studies of these enzymes in other insects is that in most cases, the starting material has not been moulting fluid, but often whole insects.

Winicur and Mitchell (1974) studied chitinase in whole *Drosophila melanogaster* larvae and pupae using a viscometric assay against a solubilized

chitosan (alkali-treated chitin) substrate. Again, the enzyme was present in highest amount at the time of moulting, but a non-negligible background was always present, and activity during the third larval stage declined only slowly after the second moult. It is possible that not all of the activity measured by the viscometric method in this study was in fact chitinase. Ammonium sulfate precipitation of the *Drosophila* chitinase suggested that more than one component was responsible for the observed activity of the extracts, although isoelectric focusing separated only one active fraction. Cation-exchange chromatography, however, revealed three peaks that had activity in the viscometric assay. Significantly, the total activity recovered was greatly reduced from that loaded, despite the enzyme's reported stability. It seems likely that this was due to the separation of synergistically acting endo- and exo-acting enzymes (see Section 3.1.1).

Spindler (1976) studied both chitinase and chitobiase (β-N-acetylglucosaminidase) activity in whole *Drosophila hydei*. The β-N-acetylglucosaminidase was assayed against a chromogenic substrate while the substrate for the chitinase was ^{14}C-labelled reconstituted chitin. The exo-acting enzyme had a high molecular weight (M_r >100 kDa), and had a much lower affinity for *p*-nitrophenyl-β-GlcNAc than did the corresponding *Manduca* enzyme. The *D. hydei* chitinase had an M_r of about 40 kDa. Both enzymes had low pH optima (pH 5.5–6.2). The enzymes occurred at high titre at the time of moulting, but the isolation methods employed led to the recovery of both enzymes not only from the integument but also from the gut and fat body. The same two enzymes were studied using the same methods in the integument of the migratory locust *Locusta migratoria* by Zielkowski and Spindler (1978), and were found to have similar properties.

Chen *et al.* (1982) purified a chitinase from whole stablefly puparia, *Stomoxys calcitrans*, to a single electrophoretic band with apparent M_r about 48 kDa. The enzyme had no cation requirement and displayed a broad pH optimum at about pH 5. The enzyme was assayed using radiolabelled acetylated chitin, and under these conditions gave a product that was almost entirely $(GlcNAc)_2$ with smaller amounts of free GlcNAc, and no detectable oligomers. These authors concluded that the enzyme they were studying might be an exo-acting chitinase.

Singh and Vardanis (1984) studied chitinase activity in the 100 000 × g supernatant of an extract of whole puparia of the housefly *Musca domestica* using an assay that released [^{3}H]-GlcNAc from labelled colloidal chitin. These authors checked that their preparations did not contain lysozyme-like activity. They found that when DEAE-cellulose purified *M. domestica* chitinase was analysed by Sephacryl S-300 gel-filtration, peaks of activity were present at 42 kDa and 120 kDa.

An interesting property of insect moulting fluid chitinases is their stability and resistance to degradation. This is also a property of fungal chitinases

(Jeuniaux, 1967). Winicur and Mitchell (1974) found that *Drosophila* chitinase was very stable (maintaining its activity for several days at room temperature, and even at 34°C). It was also notably resistant to proteolysis (withstanding a 30 min incubation with pronase). Similarly, Singh and Vardanis (1984) found that purified *Musca domestica* chitinase was highly resistant to inactivation by trypsin. These properties are significant in that *in vivo* chitinolytic enzymes are present in the moulting fluid along with an aggressive cocktail of other hydrolytic enzymes, including trypsin-like proteases. In order to remain active in this environment, chitinases would need to be resistant to proteolytic attack. Gopalakrishnan *et al.*'s (1995) demonstration that chitinase I from pharate pupal *Manduca* is extensively glycosylated may explain why this enzyme is resistant to proteolytic attack.

3.1.3 *Possible zymogenic origin of chitinase*

The work of Koga and his collaborators provides evidence that in *Bombyx mori* at least one chitinase may be synthesized as an inactive proenzyme. Antibodies raised against purified *Bombyx* chitinase (65 kDa) cross-reacted with a 215 kDa protein present in the integument two days before the appearance of active chitinase (Koga *et al.*, 1989). This was interpreted to mean that a zymogenic form of chitinase was present in the integument prior to secretion of moulting fluid. It would be particularly interesting to know if the proposed zymogen was present in the cuticle or the epidermis, but unfortunately this question was not addressed in the paper.

Further investigations of zymogenic forms of chitinase in the integument of *B. mori* and *Manduca sexta* were carried out by Koga *et al.* (1992). Polyclonal antibodies raised against *Bombyx* chitinase (88 kDa) and β-N-acetylglucosaminidase (A-B form, 125 kDa) were used to screen Western blots of integumental extracts from *Bombyx* and *Manduca*.

β-N-Acetylglucosaminidase antibodies cross-reacted with a 67.5 kDa protein in integumental extracts from both insects. The protein was detected at the SP1 stage (one day after spinning) in *Bombyx* and increased in intensity during SP2 and SP3 stages. In *Manduca* integument the protein first appeared on day 6 of the 5th instar and increased in intensity on days 7 and 8. The timing of appearance of the 67.5 kDa protein in the integument in each case coincided with the appearance of β-N-acetylglucosaminidase detected using 4-methylumbelliferol-GlcNAc activity gels.

Moulting fluid from *Bombyx* contained four β-N-acetylglucosaminidase immunoreactive proteins (67.5, 57.5, 41 and 32 kDa), whereas only one such protein (67.5 kDa) was seen in *Manduca* moulting fluid. Activity gels indicated that enzyme activity was associated only with the 67.5 kDa band, however. The role(s) of the other bands in *Bombyx* moulting fluid remains obscure. It is relevant to note that moulting fluid would probably

have contained active proteases when collected, so that the possibility of proteolytic degradation of the 67.5 kDa enzyme to produce inactive but immunoreactive fragments cannot be disregarded.

When Western blots of integumental proteins were screened using chitinase antibodies, strong cross-reaction was seen with a >200 kDa protein present in *Bombyx* integument at stages SP1, 2 and 3. At SP3 an additional immunoreactive protein (88 kDa) appeared, which coincided with extraction of active chitinase from the integument. Activity gels showed that the lower molecular weight form (but not the larger protein) possessed the ability to hydrolyse glycol chitin. These findings are similar to those of Koga *et al.* (1989) and give further evidence of zymogenic forms of chitinase. Blots of *Manduca* integumental extracts screened with *Bombyx* chitinase antibodies showed a different pattern from that seen during *Bombyx* development. A 119 kDa immunoreactive protein was seen from day 3 through to day 7 of the 5th instar and a 97 kDa protein then appeared on day 6, increasing in intensity on days 7 and 8 (two and one day before ecdysis). Chitinase activity was also first detected on day 6. It was proposed that the 119 kDa protein was a zymogenic form of chitinase in *Manduca*.

Screening of Western blots of moulting fluid with the chitinase antibody produced a different picture. Only one chitinase-immunoreactive protein (88 kDa) was seen in *Bombyx*, whereas in *Manduca* several moulting fluid proteins in the range 50 to >119 kDa cross-reacted, although the most intense band was seen at approximately 97 kDa. Koga *et al.* (1992) concluded that traces of zymogen (*c.* 119 kDa) may be present in the moulting fluid of *Manduca* but not in *Bombyx* moulting fluid. There may be real differences in the moulting process of these insects. However, it is possible that the observed differences between species were in fact due to collection of moulting fluid at different developmental stages. It is also interesting to note that there was considerably more immunoreactivity in *Manduca* moulting fluid than *Bombyx* moulting fluid (the *Manduca* gels showed much heavier staining even though only half as much moulting fluid was loaded).

However, Kramer *et al.* (1993) and Gopalakrishnan *et al.* (1995) felt confident to conclude from their molecular genetic study of *Manduca* chitinase I that there was no evidence for a high molecular weight zymogenic form of this particular enzyme. The only form of post-translational modification of the protein appears to be removal of the protein's signal sequence, followed by glycosylation, possibly in two steps (see Section 3.1.2). No combination of these modifications could yield high molecular weight forms of the enzyme like the 119 kDa protein observed by Koga *et al.* (1992). It must be concluded that the techniques used in the two laboratories must detect different proteins. It remains possible that chitinase enzymes other than chitinase I exist in zymogenic forms in *Manduca*.

Chen *et al.* (1982) addressed the question of whether *Stomoxys calcitrans* chitinase might arise as a zymogen. They noted that if this were the case,

it would be easier to explain the very rapid increase in chitinase activity that occurs immediately after puparium formation. Speculating that the low chitinase activity seen in white prepupae of this insect might be due to the lack of a proteolytic processing enzyme, they incubated preparations from this stage with trypsin, but failed to produce any increase in chitinase activity. They concluded that there was no evidence for the presence of a chitinase zymogen in *S. calcitrans*.

3.1.4 *Expression pattern of chitinase*

Using the cloned *Manduca* chitinase I cDNA, Kramer *et al.* investigated the timing and location within the insect of this gene's expression. Two types of tissue were found to express the chitinase gene, epidermis and gut. RNA from these tissues collected on day 0 through to day 8 of the 5th instar was probed with a 1.8 kb cDNA insert (Fig. 3). Chitinase mRNA was present on day 0 in gut and epidermal tissues, immediately disappearing on day 1 through to day 4. In epidermis, mRNA reappeared on day 5 and peaked on day 7, with none detected on day 8. In the gut, hybridization reappeared and peaked on day 6, subsequently declining on days 7 and 8. Chitinase activity is first detected in *Manduca* integumental extracts on day 6 (Koga *et al.*, 1992). Kramer *et al.* (1993) did not speculate as to why chitinase mRNA was present on day 0 and on day 5 in epidermal tissues, times at which no enzyme activity is detectable. It is of course possible that the chitinase mRNA may not be translatable on all occasions that it is present. It is also unclear why gut tissue produces chitinase mRNA in a similar developmental profile to the epidermis. Kramer *et al.* (1993) showed that on day 6, relative to total RNA, there is actually more chitinase mRNA in the gut than in the epidermis. It is thus possible that the gut is involved in the secretion of moulting fluid enzymes and resorption of the products of these enzymes. Alternatively, the gut chitinase may have a function that is accomplished purely within the gut. One possibility is that the gut secretes chitinolytic enzymes in order to digest the exuvia when this is eaten after ecdysis. The fact that newly moulted insects frequently (but not invariably) eat their own exuvia is well known to all who keep insects in culture. This could have the adaptive role of ensuring that all available materials within it are recovered.

3.1.5 *Absorption of chitinase onto chitin*

We have investigated the time course of changes in activity of chitinase and β-N-acetylglucosaminidase in the moulting fluid of pharate adult *Manduca sexta* (Samuels and Reynolds, 1993a). Our finding that the activities of these enzymes in the moulting fluid did not vary greatly during late pharate adult development at first sight seems to contradict the findings of a number of studies where chitinase was measured in whole integument or in old cuticle

(e.g. Jeuniaux, 1961, 1963; Winicur and Mitchell, 1974; Spindler, 1976; Bade and Stinson, 1978a), and in which a sharp pre-ecdysial peak of chitinolytic activity was found to occur. These peaks of chitinase activity may have been due to the adsorption of chitinase from the moulting fluid onto its substrate, chitin, within the old cuticle.

Bade and Stinson (1978a) examined chitinase in *Manduca* old larval cuticle during larval-pupal moulting. They found that when cuticle was incubated with moulting fluid for several hours, the cuticle developed 'endogenous' chitinase activity. Because these authors also found that chitinase was rapidly absorbed from moulting fluid onto colloidal chitin, they suggested that the appearance of chitinase activity in the cuticle was due to absorption of the enzyme from the moulting fluid. Absorption of chitinase was prevented by inhibitors of a trypsin-like enzyme present in the moulting fluid. Moreover, absorption was enhanced when cuticle was deproteinized before incubation with moulting fluid. It was suggested that a trypsin-like enzyme present in the moulting fluid acted to promote absorption by revealing endogenous chitin onto which chitinase then could be absorbed. In accord with this, these authors found a sharp peak of bound chitinase in the old larval cuticle at about the time of most rapid cuticle degradation (unfortunately timing was not more precisely established relative to ecdysis). It seems very likely that this peak represented chitinase which was removed from the moulting fluid by absorption onto newly revealed chitin in the cuticle.

With these findings in mind, it is necessary to be cautious in concluding that there is no sharp peak of moulting fluid chitinase activity preceding the eclosion of adult *Manduca*. If chitinase is continuously being removed from the moulting fluid by adsorption onto the old cuticle, then it may be that the enzyme level in moulting fluid alone is not a measure very relevant to the process of chitin degradation in the old cuticle. However, the conclusions (Samuels and Reynolds, 1993a) that chitinase is clearly present in the moulting fluid well before degradation of the old cuticle begins, and that the onset of this degradation is heralded by the appearance of proteolytic enzymes in moulting fluid, remain secure. This may also apply to the larval-pupal moult in *Manduca*. Koga *et al.* (1992) show that active chitinase is present in the integument during the three days that precede pupal ecdysis, even though (Truman *et al.*, 1980) the old larval cuticle is not degraded until the last 24 h before ecdysis. Thus, whether or not chitinase activation is the result of the cleavage of a zymogen, the access of the activated enzyme to its substrate does not occur until considerably later.

3.2 PROTEOLYTIC ENZYMES

Although proteases were the first enzymes to be shown as present in moulting fluid (Wigglesworth, 1933), studies of these enzymes have subsequently been less numerous than those examining chitinolytic enzymes.

Following the pioneering work of Passoneau and Williams (1953) on the moulting fluid of pharate adult *Hyalophora cecropia* which demonstrated the presence of protease activity, more detailed studies on *Antheraea polyphemus* showed the presence of two trypsin-like enzymes (Katzenellenbogen and Kafatos, 1970, 1971a). Although it was shown that moulting fluid could digest pupal cuticle, the presumption that this was due to the presence of the two trypsin-like enzymes was not directly tested, except to show that pure bovine trypsin could also solubilize cuticle.

Bade and Shoukimas (1974) described a trypsin-like serine protease and a neutral metal chelator-sensitive protease from the moulting fluid of the tobacco hornworm *Manduca sexta*. It was shown (Bade and Stinson, 1978a) that inhibition of the serine protease prevented the adsorption of chitinase from moulting fluid into the cuticle, and it was hypothesized that the function of the trypsin-like enzyme was to degrade cuticle proteins and thus render cuticle chitin susceptible to attack by chitinases. Brookhart and Kramer (1990) recognized the presence of numerous proteolytic activities in pupal *M. sexta* moulting fluid.

However, in none of these cases was the ability of a moulting fluid protease to degrade its natural substrate, cuticle, directly demonstrated. We have attempted to remedy this deficit in our work with *M. sexta* pharate adult moulting fluid (Samuels *et al.*, 1993a,b). Two proteolytic enzymes have been purified and partially characterized. One of these is a proteinase (i.e. an endo-acting enzyme that can degrade proteins by cleavage at sites within the amino acid chain). Another is an aminopeptidase (i.e. an exo-acting enzyme that acts to remove terminal residues from the N-terminal end of the peptide chain).

3.2.1 *Proteinases*

Samuels *et al.* (1993a) purified and characterized a 41 kDa cuticle-degrading proteinase from the moulting fluid of *M. sexta*, designating the enzyme Moulting Fluid Protease 1 (MFP-1). This enzyme may be the same as the trypsin-like serine protease found in pharate pupal moulting fluid by Bade and Shoukimas (1974), but appears to have rather different properties to those described for enzymes in pharate pupal moulting fluid by Brookhart and Kramer (1990). MFP-1 can be classified as trypsin-like from its substrate specificity and because it is inhibited by the trypsin inhibitor from soybean (SBTI). However, MFP-1 is more specific in its requirements than trypsin. A classical trypsin specificity would be directed towards both Lys and Arg internal residues. In fact, MFP-1 shows a pronounced primary specificity for elongated substrates with arginine at the P_1 position, the substitution of lysine at this position resulting in an 86% reduction in activity. This type of specificity was also found in the trypsin-like enzymes (30 kDa and 34 kDa) from silkmoth moulting fluid documented by Katzenellenbogen and Kafatos

(1971a), and in a serine proteinase isolated from *Drosophila melanogaster* embryos (Medina and Vallejo, 1989). By far the best substrate of those tested by Samuels *et al.* (1993a) for MFP-1 activity was Bz-Gly-Pro-Arg-NH_2. The substrate specificity of MFP-1, and its susceptibility to inhibition by the very specific inhibitor hirudin, indicates that this enzyme is functionally similar to the mammalian blood-clotting enzyme, thrombin. Whether this similarity has any evolutionary or functional significance remains to be seen.

Active site classification of MFP-1 is not straightforward. Although MFP-1 is inhibited by the classical active site serine inhibitors DFP and PMSF, it is also inhibited by the diagnostic inhibitor of cysteine proteinases, E-64. These characteristics are not common but have been found in other proteinases (see Beynon and Bond, 1989; R. J. St Leger, Boyce Thompson Institute, Cornell University, personal communication).

MFP-1 is the major cuticle-degrading enzyme found in the moulting fluid of pharate adult *Manduca sexta*. MFP-1 was shown to degrade *Manduca* cuticle proteins *in vitro*, producing polypeptide fragments in the size range 200–2000 Da (Samuels *et al.*, 1993b). These peptides are presumably substrates for subsequent digestion by less specific enzymes, e.g. the aminopeptidase MFP-2.

The activities of moulting fluid proteinases from *M. sexta* pharate pupae were investigated by Brookhart and Kramer (1990). These authors succeeded in only a limited degree of purification of these enzymes. Whole moulting fluid contained an atypical tryptic-like enzyme that hydrolysed the substrate benzoyl arginine ethyl ester (BAEE) but not benzoyl arginine *p*-nitroanilide (BAPNA). Some activity of a chymotryptic type (against benzoyltyrosine ethyl ester [BTEE], and benzoyltyrosine *p*-nitroanilide [BTPNA]) was present, but at a much lower level. It is not clear whether these activities represent the same or different enzymes, but neither is very like MFP-1.

Brookhart and Kramer (1990) found a Sephacryl S-300 fraction that hydrolysed azocoll (M_r about 100 kDa), but which was apparently not identical with the major fraction cleaving gelatine, which was slightly larger. The azocoll-cleaving fraction had activity against BAEE, but incubation with a model hexapeptide substrate (Leu-Trp-Met-Arg-Phe-Ala) indicated that this fraction contained one or more enzymes with both tryptic and chymotryptic specificities. It is not clear whether this represented incomplete purification, or whether one enzyme with multiple specificities was present. Inhibitor studies were not much help. Activity of this fraction was not substantially inhibited by DFP, PMSF or diagnostic inhibitors of cysteine proteases. Brookhart and Kramer (1990) suggested that the presence of a multicatalytic proteinase might be responsible for these unusual results. This normally intracellular enzyme, now known as the proteasome, has received much attention in recent years (reviewed by Peters, 1994), and it appears that the enzyme may not only be involved in the extralysomal pathway of protein turnover (Ciechanover and Schwartz, 1994), but that it may also have

a special role in the programmed cell death of *Manduca* intersegmental muscles at the time of adult ecdysis (Dawson *et al.*, 1995; L. M. Schwartz, University of Massachusetts, Amherst, personal communication). It may be appropriate to note here that we (Samuels and Reynolds, 1993a) found that before treatment with detergent, MFP-1 in the moulting fluid of pharate adult *Manduca* had an apparently very high molecular weight. This indicates that in its native state MFP-1 may be part of a larger enzyme complex.

It is at least clear that the moulting fluid proteinases of *Manduca* are not classical trypsins. Classical trypsin-like enzymes have been isolated from insects, for example, the midguts of *M. sexta* larvae (Miller *et al.*, 1974) and of Vespa spp. (Jany *et al.*, 1978). cDNAs encoding trypsinogen-like enzymes have been cloned from *D. melanogaster* (Davis *et al.*, 1985), *M. sexta* (Peterson *et al.*, 1994), and *Lucilia cuprina* (Casu *et al.*, 1994), which are thought to represent the precursors of gut trypsins. In each case there are multiple genes in each species. Insect gut trypsins have classical trypsin-like specificities, being inhibited by diagnostic inhibitors of serine proteinases and the normal range of substrate inhibitors (e.g. SBTI, leupeptin, TLCK). Their inferred sequences are conventional and clearly related to the trypsins of other animal digestive systems. Unfortunately, no sequence data are yet available for the moulting fluid enzymes. It will be of considerable interest to compare the sequences of the trypsin-like serine proteases from moulting fluid with the corresponding gut enzymes. A conclusion which may already be drawn, however, is that if the gut does in fact contribute to the elaboration of the moulting fluid as discussed in Section 2.1, then it is not by simply secreting the normal range of digestive enzymes. In fact, like other animals, insects elaborate large numbers of serine protease family enzymes, expressing them differentially in different cell types for particular purposes. First-instar *Lucilia* larvae are estimated to express between 125 and 220 different serine protease genes (Elvin *et al.*, 1994). At least some of these may represent specialized moulting fluid proteinases.

No chymotrypsin-like activity was detected in the moulting fluid of pharate adult *M. sexta* (Samuels *et al.*, 1993a), and although chymotrypsin-like activity was detected by Brookhart and Kramer (1990) in semi-purified fractions from *M. sexta* pharate pupal moulting fluid, these investigators found that the activity of chymotrypsin-like enzymes was tenfold lower than that of trypsin-like enzymes. It may be that chymotrypsin-like enzymes are in general not very important in insect extracellular protein-degrading systems. A similarly low ratio of chymotrypsin to trypsin activity was measured in the midgut of the grass grub *Costelytra zealandica* (Christeller *et al.*, 1989). Casu *et al.* (1994) found that although feeding trypsin inhibitors was very deleterious to *Lucilia cuprina*, chymotrypsin inhibitors had no effect. It is possible, however, that the choice of substrates and inhibitors may be important in this kind of study than has been realized in the past. Working with *Spodoptera* larval gut contents, Lee and Anstee (1994) found

a chymotrypsin-like enzyme that had a requirement for N-terminally extended substrates. This enzyme did not hydrolyse a number of classical chymotrypsin substrates and was not inhibited by some traditional chymotryptic inhibitors.

3.2.2 *Peptidases*

Bade and Shoukimas (1974) were the first to report a neutral metal-chelator-sensitive protease in the moulting fluid of *M. sexta* pupae. A similar exo-cleaving, divalent ion-dependent aminopeptidase activity (apparent M_r 500 kDa) was detected in the moulting fluid of pharate pupal *Manduca* by Brookhart and Kramer (1990), although the enzyme was not purified. Samuels *et al.* (1993b) purified and partially characterized an aminopeptidase from the moulting fluid of pharate adult *M. sexta* which was designated as MFP-2. It is probable that all of these papers describe the same enzyme. Samuels *et al.* (1993b) found that the enzyme has a native molecular mass of 240 kDa which comprises six subunits of 40 kDa. The native M_r is similar to that stated for a leucine aminopeptidase (280 kDa) isolated from *D. melanogaster* by Walker *et al.* (1981). The *Drosophila* aminopeptidase activity was found to vary with developmental stage, which led Hall (1988) to suggest a moulting-related role for the enzyme. During the later stages of pre-ecdysial development in pharate adult *M. sexta*, the activity of MFP-2 in moulting fluid rises steadily, reaching a maximum approximately 16 h prior to ecdysis (Samuels and Reynolds, 1993a). The activity of this enzyme was not affected by injections of 20-hydroxyecdysone, unlike MFP-1 (Samuels and Reynolds, 1993b), a further indication of the secondary role of MFP-2.

MFP-2 degrades a broad range of aminoacyl substrates with a preference for methionine-, leucine- and alanine-β-naphthylamides. On the basis of inhibitor studies this enzyme was classified as a metallopeptidase. Ion replacement studies suggested that the metal ion involved was cobalt rather than the more common zinc. MFP-2 failed to degrade insect cuticle when used alone. However, when MFP-2 was applied to insect cuticle together with MFP-1, the amount of free amino acids released was significantly greater than that seen for MFP-1 alone (Samuels *et al.*, 1993b). The combined action of these two enzymes resulted in the cuticular proteins being reduced to oligopeptides and free amino acids which could presumably be more readily reabsorbed by the insect.

3.3 OTHER MOULTING FLUID ENZYMES

Other enzymes occur in moulting fluid, but their functions are obscure.

Katzenellenbogen and Kafatos (1970; 1971c) found that moulting fluid of pharate adult silkmoths contained two distinct esterases that hydrolysed

esters of aromatic alcohols. These were characterized as carboxylesterases because of their activities towards 1-naphthyl acetate and their sensitivities to inhibitors.

Mai and Kramer (1983) purified three similar esterases from the pharate pupal moulting fluid of *Manduca sexta*. None of these enzymes had any hydrolytic activity towards protein substrates. Two of the enzymes (EI and EII) were carboxylesterases with properties similar to the enzymes described by Katzenellenbogen and Kafatos (1971c). EI also cleaved choline esters and was partially inhibited by eserine, and EIII hydrolysed juvenile hormone. These properties led the authors to suggest that these enzymes possessed some of the properties of acetylcholine esterase and juvenile hormone esterase respectively. Whereas EIII appeared to be a single protein, EI could be shown to be comprised of at least three separate peaks on activity gels. Both enzymes could be inhibited by the organophosphate diisopropylfluorophosphate (DFP). The other esterase, EII, was also apparently a single protein. It was inhibited by mercurials, but not DFP. The authors described it as an arylesterase or 'A-esterase'. The endogenous substrates for all of these enzymes are unknown and their *in vivo* functions remain unproven. Mai and Kramer (1983) suggested that they might 'metabolize esters found in cuticular lipids, waxes and hormones'.

Schneiderman *et al.* (1966) and Knowles and Fristrom (1967) found an alkaline phosphatase activity in the integument of *Drosophila melanogaster* that was distinct from other isozymes found in other tissues. This peaks in late third instar larvae preparing for pupariation. Psarianos *et al.* (1987) found two alkaline phosphatases in the integument of the Mediterranean fruitfly *Ceratitis capitata*. One of these appeared in the integument just prior to eclosion, while the other was present in the mid-pupal stage. These bands were distinct from another alkaline phosphatase present in the haemolymph. It is not clear whether any of these enzymes occurs in the moulting fluid.

We (S. E. Reynolds and A. M. Brown, unpublished) have found that pharate adult *Manduca sexta* moulting fluid contains both acid phosphatase and β-glucuronidase activity. These enzymes are usually considered to be lysosomal markers, and their presence in the moulting fluid may indicate a lysosomal origin for moulting fluid enzymes. However, it is doubtful if acid phosphatase and β-glucuronidase are functional as cuticle-degrading enzymes, as both enzymes have distinctly acid pH optima, while the moulting fluid's pH is on the alkaline side of neutrality (see Section 2.1).

Saito (1993) studied a biliverdin binding protein (BBP) from moulting fluid of pharate pupae of the saturniid silkmoth *Samia cynthia ricini*. The protein was purified using successive hydrophobic-interaction and gel-filtration chromatography. The final gel permeation HPLC step showed the protein to have an M_r of 49 kDa, whereas SDS-polyacrylamide gel electrophoresis gave a figure of 24 kDa, indicating that the protein was probably a dimer *in vivo*. There was no apparent homology with biliverdin binding proteins

of other insects (e.g. insecticyanin from *M. sexta*). The *Samia* BPP is particularly interesting because, except for the moulting fluid, it is found only in the insect's cuticle, and is absent from haemolymph and even epidermis. Presumably the protein is solubilized during moulting, and is then resorbed, to be recycled directly into the new cuticle. Saito (1993) pointed out that this protein might be an excellent marker for localized resorption of moulting fluid materials. The question of why this protein is not degraded by the proteolytic enzymes of the moulting fluid has not yet been addressed.

3.4 ENZYME ACTIVITY AND THE MOULTING CYCLE

As would be expected, the correspondence between the occurrence in the integument of chitinase and proteinase enzyme activities was recognized early in the field of moulting research. Jeuniaux (1961; 1963) showed that chitinase activity in the integument of the silkmoth *Bombyx mori* peaked just before the time of ecdysis at both larval–larval and larval–pupal moults. The enzyme became detectable at apolysis and increased in activity as ecdysis approached. This was confirmed for *Bombyx* larvae by Kimura (1976). Chitinase disappeared at ecdysis. Similar pre-ecdysial peaks of chitinase activity have been seen in a number of other insects, including *Drosophila melanogaster* (Winicur and Mitchell, 1974); *Manduca sexta* (Bade and Stinson, 1978a) and *Locusta migratoria* (Zielkowski and Spindler, 1978).

In pupae of *Stomoxys calcitrans* (Chen *et al.*, 1982), and *Musca domestica* (Singh and Vardanis, 1984), however, the change in chitinase activity followed a very different trajectory. Chitinase was detectable very soon after puparium formation, rose slowly to a mid-pupal peak, and declined slowly again prior to eclosion of the adult fly. This raises the question of why cuticle digestion does not occur sooner than it does in these dipteran pupae.

Interestingly, where the question has been examined, it seems that an increase in β-N-acetylglucosaminidase activity precedes the premoult rise in chitinase. This is true for both *Bombyx mori* (Kimura, 1973; 1977; Koga *et al.*, 1989) and *Locusta migratoria* (Zielkowski and Spindler, 1978). Koga *et al.* (1989) concluded that this must mean that the hydrolysis of cuticular chitin is initiated not by the rise in β-N-acetylglucosaminidase, but by the later increase in chitinase activity. As will be discussed below (Section 3.5.1) they suggested that the latter was a consequence of the activation of a previously synthesized chitinase zymogen. Some representative data for the activities of chitinolytic enzymes during the moult cycle in various insects are shown in Fig. 8.

The only cases in which moulting fluid proteolytic activity has been followed in relation to the progress of moulting, are the saturniids *Antheraea polyphemus* and *Hyalophora cecropia* (Katzenellenbogen and Kafatos, 1970) and the sphingid *M. sexta* (Samuels and Reynolds, 1993a). In each case,

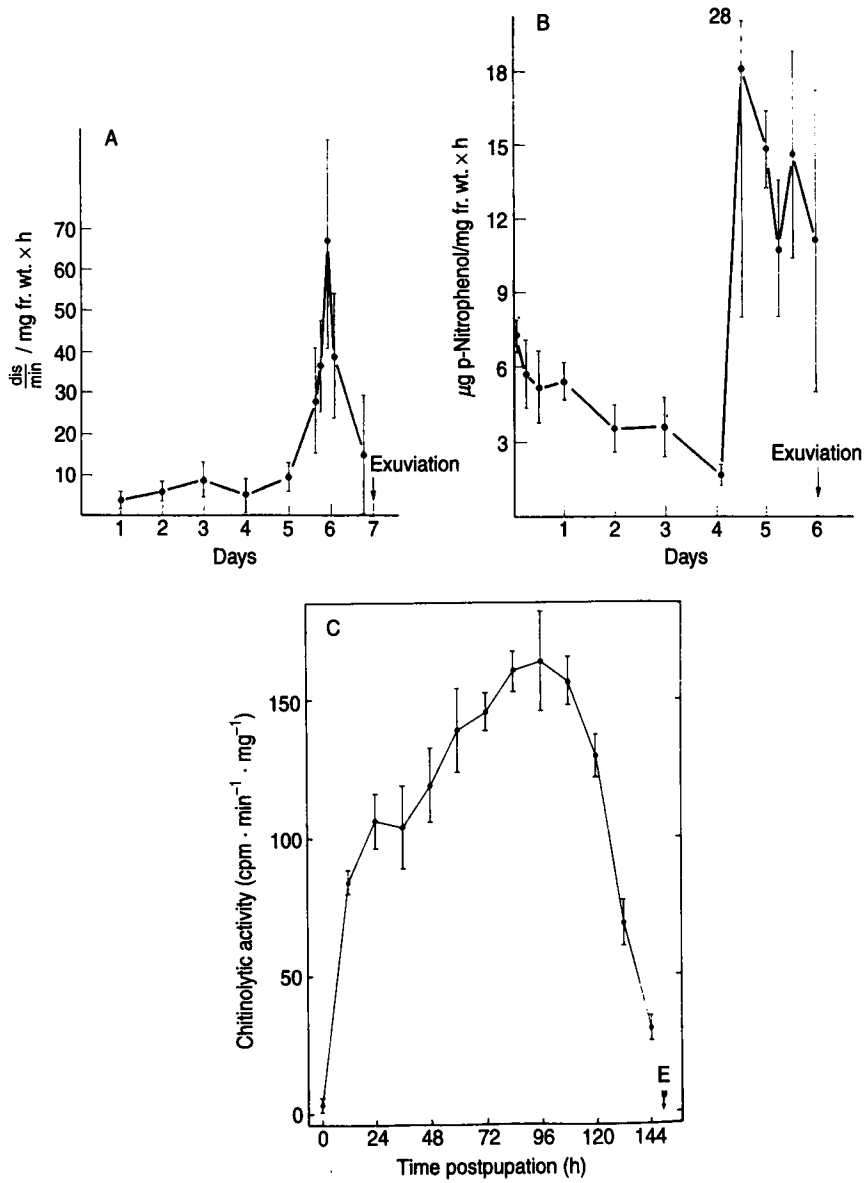

FIG. 8 Chitinolytic activity during the moult cycle in locusts and flies. (A) Chitinase activity in fifth-stage larvae of *Locusta migratoria*; (B) β-N-acetylglucosaminidase (chitobiase) activity in *L. migratoria* (A and B from Zielkowski and Spindler, 1978); (C) Chitinase activity during the pupal stage of *Stomoxys calcitrans* (from Chen *et al.*, 1982).

pharate adults were studied. The pattern of changing proteolytic activity is somewhat different in these two lepidopteran Orders.

In pharate adult *A. polyphemus*, trypsin-like proteinase activity (determined using both gelatine film and also N-α-benzoyl-L-arginine ethyl ester [BAEE] as substrates) is undetectable before up to day 11, appears first on day 12, peaks on day 14, and subsequently declines somewhat before adult ecdysis on day 17 (Katzenellenbogen and Kafatos, 1970). This corresponds well with the timing of pupal cuticle degradation, which is most rapid on days 14 and 15. In *H. cecropia*, where adult development takes longer (21 days), a peak of trypsin-like proteinase activity is seen on day 14–16 which declines thereafter. By day 18–19, the level of activity in the moulting fluid of this species is only half that seen at the peak. This corresponds with the longer period spent by this insect in the cocoon with an apparently fully digested pupal endocuticle.

In pharate adult *Manduca* things are different. Here, the serine proteinase MFP-1 increases in activity dramatically during the 100 h preceding adult eclosion, and this increase parallels the increased ability of the moulting fluid to degrade pupal cuticle (Fig. 9) (Samuels and Reynolds, 1993a). Initially undetectable, MFP-1 activity increases at the time that the old pupal cuticle begins to thin, and the maximum rate of thinning is achieved at the time that MFP-1 activity is highest. The aminopeptidase MFP-2 also increases in activity during this period, although the increase is not so great as that of MFP-1. In this species there is no decline in proteolytic activity prior to eclosion. This is true for the release of radioactivity from prelabelled cuticle, and also for assays of MFP-1 and MFP-2 using chromogenic substrates. This corresponds to the much later timing of pupal cuticle breakdown in pharate adult *M. sexta* compared with the silkmoths studied by Katzenellenbogen and Kafatos (1970). Although some loss of weight from the old cuticle is seen as early as about 80 h before ecdysis, rapid cuticle thinning is seen only in the last few hours before ecdysis (see Fig. 9).

The relationship between the timing of appearance of chitin- and protein-degrading enzymes is interesting. Samuels and Reynolds (1993a) measured both proteolytic and chitinolytic activity in moulting fluid from pharate adult *M. sexta*, and correlated this with cuticle thinning. Both chitinase and β-N-acetylglucosaminidase were present in moulting fluid long before the pupal cuticle began to be broken down. The onset of thinning was not correlated with changes in the activity of either one of these enzymes, but did correspond quite well to the appearance in the moulting fluid of the two proteolytic enzymes, MFP-1 and MFP-2. In fact, the activities of the chitin-degrading enzymes did not vary appreciably at all during all the time that moulting fluid could be collected. This suggests that the timing of breakdown of the old cuticle is determined not by chitinolytic activity, but by proteases.

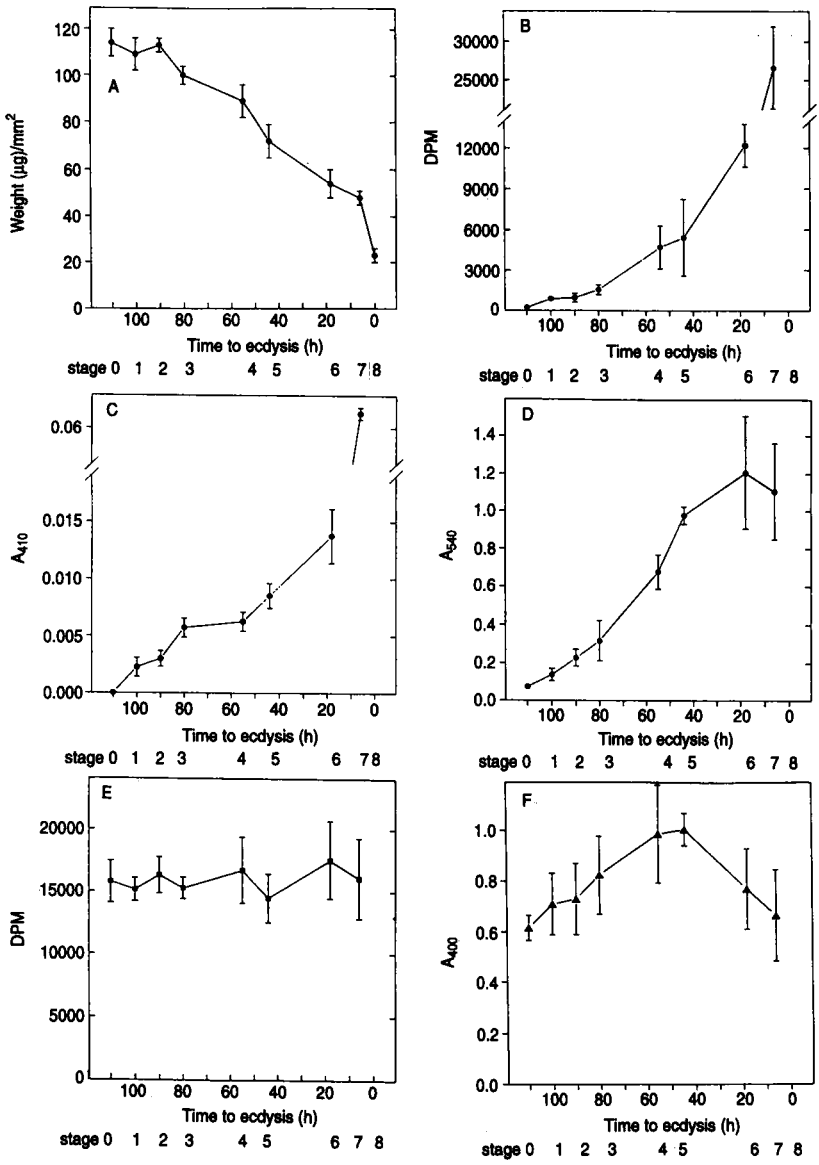

FIG. 9 Activities of moulting fluid enzymes during the period preceding adult eclosion in *Manduca sexta*. (A) Thinning of the old cuticle. Discs of pupal cuticle were obtained from pharate adult insects during each stage of development and thickness expressed as mean fresh weight ($\mu g\,mm^{-2}$). (B) Cuticle degrading activity, expressed as the ability of whole moulting fluid to release soluble radioactive material from cuticle biosynthetically labelled with [^{3}H]-leucine. Note line break on vertical axis. (C) MFP-1 activity. Assayed using Tosyl-glycyl-prolyl-arginyl-*p*-nitroanilide as substrate, activity expressed as increase in A_{410} per 5 min. (D) MFP-2 activity.

3.5 ACTIVATION OF MOULTING FLUID ENZYMES

3.5.1 *Chitinolytic enzymes*

As noted in Sections 2.1 and 3.4, the moulting fluid is not active in degrading the old cuticle when it is first secreted. Passoneau and Williams (1953) found that in pharate adults of *Hyalophora cecropia* secretion of the moulting gel preceded activation of protease and chitinase by 14 days, and that the fluid is only enzymatically active for 5 days during which time the old cuticle is degraded. Our own results with pharate adult *Manduca sexta* (Samuels and Reynolds, 1993a) tell a similar story (see Fig. 9). The presumption (discussed above) that the enzymes which are present in the moulting gel/fluid are synthesized by the cuticular epidermis (perhaps also by the gut epidermis) requires either that these enzymes are produced and secreted as inactive forms (pro-enzymes; zymogens), and that these are activated only much later, or that the enzymes are inhibited or otherwise unable to gain access to the substrate prior to 'activation'.

Chitinolytic enzymes elaborated in the epidermis or gut as active or inactive forms may be secreted with the moulting gel/fluid, or transported into the 'old' cuticle prior to apolysis. Two scenarios for the subsequent 'activation' of chitinolytic enzymes may be entertained. First, as discussed above, it has been suggested by Koga *et al.* (1989) (but not supported by the molecular genetic work of Kramer *et al.*, 1993), that some or all of the chitinases present in the moulting fluid are secreted in the form of inactive proenzymes. These enzymes would then be activated by limited proteolysis at some later time. Thus the onset of chitin degradation would be signalled by the secretion of an activating protease. The alternative to this model (the two are not necessarily mutually exclusive) is that moulting fluid chitinases are potentially active as soon as they are secreted but are unable to gain access to their substrate, chitin.

In our work with pharate adult *Manduca* we have found that active chitinase and β-N-acetylglucosaminidases can be detected in the moulting fluid during all stages of the moult, even before cuticle thinning begins, and that the activities of these enzymes do not increase as cuticle thinning occurs (Samuels and Reynolds, 1993a). In the migratory locust, *Locusta migratoria*,

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Assayed using L-alanyl-β-naphthylamide as substrate, activity expressed as increase in A_{540} per 10 min. (E) Chitinase. Assayed as release of soluble radioactivity from [^{3}H]-labelled colloidal chitin, activity expressed as DPM released per 30 min. (F) β-N-acetylglucosaminidase. Assayed using p-nitrophenyl-N-acetylglucosamine as substrate, activity expressed as increase in A_{400} per 10 min. In each case, blanks were subtracted from data. Means ± S.D. ($n = 5$). From Samuels and Reynolds (1993a).

the resorption of cuticular proteins precedes resorption of chitin (Phillips and Loughton, 1976). These findings are in general agreement with the hypothesis first advanced by Bade and Stinson (1978b), and subsequently reiterated by others (Fukamizo and Kramer, 1985b; Smith *et al.*, 1981; St. Leger *et al.*, 1986b), that protease activity is required in order to allow chitinolytic enzymes to act.

We have suggested (Samuels and Reynolds, 1993a) that active chitinases are present in the moulting fluid before the appearance of proteolytic activity, but are unable to gain access to chitin microfibres which are protected by the surrounding matrix proteins. Chitinase would thus only be able to interact with chitin when these proteins had been degraded by proteolytic enzymes. This hypothesis is supported by the simple observation that whereas the chitin in fungal cell walls is readily stained with chitin-specific fluorescent dyes such as Calcofluor M2R or fluorescein isothiocyanate-wheatgerm agglutinin (FITC-WGA), the chitin of intact insect cuticle is not. The latter only becomes stainable when it has been partially digested with proteases, presumably because the chitin microfibres are physically shielded by surrounding matrix protein. It is this that allows the progress of infection by insect pathogenic fungi (such as *Metarhizium anisopliae*) to be followed by the specific staining of their hyphae by Calcofluor (Kritzman *et al.*, 1978). St Leger *et al.* (1986b) found that pieces of desert locust (*Schistocerca gregaria*) cuticle could be stained with Calcofluor or FITC-WGA only at the cut edges. After exposure to cultures of *M. anisopliae*, however, the cuticle became generally stainable, presumably because the cuticle's protein matrix had been digested away by the fungal cuticle-degrading enzyme Pr1. Interestingly, exuvia and highly sclerotized cuticle were resistant both to Pr1 and also to chitin-specific staining.

In this regard it is interesting that there may be specific associations between certain cuticle proteins and chitin. These proteins are very difficult to solubilize, remaining with the 'chitin' fraction even after extensive extractive treatment (short of the alkaline hydrolysis usually used to prepare chitosan). Nakato *et al.* (1994) have reported the isolation of chitin binding proteins from larval and pupal cuticle of *Bombyx*, by means of chitin affinity chromatography. Andersen *et al.* (1995) have suggested that a 28-residue hydrophilic consensus sequence present in 20 cuticular proteins from four different Orders of insects might be involved in this association with chitin. It is possible that such proteins might protect chitin from attack by chitinases, and might be attacked by specific proteinases in order to initiate general cuticular degradation.

3.5.2 *Proteolytic enzymes*

At least some of the cuticle-degrading proteinases present in the moulting fluid are probably secreted into the moulting fluid as inactive forms at an

earlier stage of the moulting process. When the pharate adult epidermis of saturniid silkmoths retracts from the overlying pupal cuticle, a colourless gel fills the exuvial space. Once the new adult cuticle has formed, the moulting gel becomes the less viscous 'moulting fluid' and cuticle degradation begins (Passoneau and Williams, 1953; Katzenellenbogen and Kafatos, 1970). Katzenellenbogen and Kafatos (1971b) have shown that *Antheraea polyphemus* moulting gel contains inactive proteolytic enzymes which become active *in vitro* by dilution with buffer of low molarity.

One explanation for the finding that the moulting fluid is initially inactive that was explored by Katzenellenbogen and Kafatos (1971b) is that the cuticle-degrading enzymes may be compartmentalized in the moulting gel, so that they are not available to attack the substrate. Relevant to this hypothesis are the many observations of cells ('haemocytes') within the moulting fluid (see Section 2.2). It is possible that dilution of the moulting fluid leads to the osmotic lysis of these cells, and the liberation of cuticle-degrading enzymes within them. We find such an explanation to be improbable, since these cells occupy only a minute fraction of the moulting fluid, and it seems very unlikely that all the necessary enzymes for the degradation of the old cuticle would be contained within them.

A second explanation that seems more likely is that inactive zymogens in the moulting fluid could be activated by the liberation of a specific zymogen-activating protease (or more than one such enzyme) from cells in the moulting fluid. This could account for Katzenellenbogen and Kafatos's (1971b) finding that once activated by dilution, the moulting fluid proteases could not be deactivated by concentrating the moulting fluid again. Once released, tiny amounts of this activating enzyme could activate all the inactive zymogens already present. Here again is a parallel with the situation in the mammalian gut, where trypsin is secreted as the inactive trypsinogen, and is subsequently activated first by enterokinase, a specific proteinase present in the wall of the small intestine. It is interesting that in *M. sexta*, there is no evidence of such an enterokinase-like system in the activation of gut trypsins (Peterson *et al.*, 1994), but this does not mean that such a system could not operate in the moulting fluid.

One of Katzenellenbogen and Kafatos's (1971b) experiments indicates an important difference between the moulting fluid enzymes and the gut trypsin system, however. It was found that the process of protease activation on dilution of moulting fluid was a strictly first-order reaction, so that autoactivation cannot be an important feature of the process. This contrasts strongly with the gut where limited-proteolytic autoactivation of trypsin occurs once enterokinase has begun the process. This conclusion is reinforced by Katzenellenbogen and Kafatos's (1971b) finding that already-active moulting fluid failed to activate the inactive proteases of moulting gel. This mixture would have been expected to gain activity if autoactivation occurred. The further conclusion from this experiment

must be that the activated moulting fluid no longer contained the factor that had activated it.

A third possibility for the regulation of cuticle-degrading enzymes would be the secretion into the moulting fluid at the time of its formation of specific inhibitors together with the enzymes. It is possible that such an inhibitor might be diluted out on addition of buffer to the moulting gel. Katzenellenbogen and Kafatos (1971b) argued against this by pointing out first that reconcentration of already active moulting fluid did not lead to loss of activity, and second that addition of moulting gel to active moulting fluid did not inhibit the latter. However, an interesting possibility that was not considered is that the postulated inhibitor might not inhibit the cuticle degrading proteinase itself, but instead an activating enzyme that acts to cleave, and thus activate a zymogen. Such a system would be expected to respond in a highly non-linear and irreversible way to the dilution of inhibitor.

Although Katzenellenbogen and Kafatos did not find a protease inhibitor in silkmoth moulting fluid, we (R. I. Samuels and S. E. Reynolds, unpublished) have found that the moulting fluid of pharate adult *M. sexta* contains proteinase inhibitors active against trypsin- and chymotrypsin-type enzymes. We did not find it possible to activate moulting fluid proteases by dilution of moulting fluid. However, at least one of the inhibitors that we found is active against MFP-1. We separated the inhibitor from MFP-1 by passage of moulting fluid through a Heparin affinity column, which retained the enzyme, but not the inhibitor. The inhibitor masks the enzyme's presence prior to this separation step, so that the yield of active MFP-1 is greatly increased when this inhibitor is removed during purification (Samuels *et al.*, 1993a). Preliminary results showed that the content of the MFP-1 inhibitor in moulting fluid fell during the later stages of ecdysis, indicating a possible regulatory role in the 'activation' of MFP-1.

Actually, *Manduca* haemolymph contains numerous types of proteinase inhibitor (Kanost, 1990). At least 12 different 47 kDa serine protease inhibitors (serpins) are generated from the *Manduca* serpin-1 gene by alternative splicing to generate variants with potential to inhibit different proteinases (Jiang *et al.*, 1994). Recently, it has been found that the titre of these haemolymph serpins falls at the time of ecdysis (Kanost *et al.*, 1995). Northern blots showed that the serpin-1 gene is transcribed in fat body and haemocytes, but not other tissues. In larval instars serpin-1 mRNA disappeared abruptly from fat body cells at around the time of apolysis, reappearing after ecdysis. However, the pattern of inhibitor level in the haemolymph, and of its mRNA in fat body, did not fit this pattern during pupal and adult stages. The authors suggested that one or more of these serpins might have some role in the moulting process, although they did not speculate what this might be, nor whether the inhibitor could be translocated from the haemolymph to the moulting fluid (remember that haemocytes may be present in moulting fluid – see Section 2.2.1).

It should be borne in mind that insect proteinase inhibitors are likely to have additional functions unconnected with moulting. One such additional role for proteinase inhibitors in the moulting fluid may be as regulators of the enzymes that activate phenoloxidase, as in the haemolymph (Boigegrain *et al.*, 1992; Tsukamoto *et al.*, 1992). Another would be in defence of the insect against invasion by potentially harmful micro-organisms, such as entomopathogenic fungi. The chymotrypsin inhibitor present in *Manduca* moulting fluid has a high affinity for the fungal proteinase Pr1 (R. I. Samuels, unpublished data). A proteinase inhibitor has also been isolated from the haemolymph of *Bombyx mori* which has a high affinity for fungal proteinases (Eguchi *et al.*, 1993). A proteinase inhibitor is present in crayfish cuticle, which may provide some protection against penetration by invading micro-organisms (Hall and Soderhall, 1983).

It remains possible that some or all of the observed changes in proteolytic enzyme activity in pharate adult *M. sexta* moulting fluid may be the result of changes in the *de novo* synthesis of the enzymes. This possibility is supported by the finding (R. I. Samuels, unpublished observations) that removal of all available moulting fluid (*c.* 50% of total) from pharate adult *Manduca* (24 h–48 h before ecdysis) had no apparent adverse effects on subsequent ecdysis. The insect was able to replace the lost fluid within 6 h. The cuticle-degrading activities of moulting fluid from the first collection and from the replaced fluid (6 h) were similar. This may imply that there is a continuous flow of moulting fluid both into and out of the exuvial space at this time.

3.6 CUTICLE-DEGRADING ENZYMES FROM INSECT PATHOGENIC FUNGI

The moulting fluid's task is to degrade the old cuticle, rescuing from it the raw materials from which the new cuticle can be constructed. This job specification is very similar to that of the enzymes secreted by an entomopathogenic fungus as it invades its host through the cuticle (Charnley and St Leger, 1991). It therefore seems appropriate to compare the two. We will do so only briefly, because the subject has been reviewed elsewhere (Charnley and St. Leger, 1991; St Leger, 1991; Samuels and Paterson, 1995). It will be seen that there are certain differences between the ways in which the fungus and the insect achieve their respective goals.

The ability of entomopathogenic fungi to gain entrance to their hosts through the cuticle has long been known, and this suggested that these fungi must secrete cuticle-degrading enzymes during the infection process. Gabriel (1968) was the first to show the production of suitable enzymes by entomophthoralean fungi in culture, including chitinolytic, proteolytic and lipolytic activities. Subsequently similar studies were made of the deuteromycetes *Beauveria bassiana* (Leopold and Samsinakova, 1970) and *Metarhizium anisopliae* (Kucera, 1980).

The work of St. Leger and his associates has shown that entomopathogenic

fungi produce a wide range of proteinases and peptidases in culture, and that these enzymes are highly effective cuticle-degrading agents. The case of *M. anisopliae* is the best studied. Cultures of this fungus produce cuticle-degrading enzymes *in vitro* when cultured on ground locust cuticle (St. Leger *et al.*, 1986a). Four distinct proteinase activities have been identified, of which three enzymes have been extensively characterized. Several of the enzymes appear to occur as isoenzymes, but this will not be discussed further here. Pr1 and Pr2 are both serine proteinases. Pr1 is described as a chymoelastase, and possesses a broad specificity for synthetic substrates, cleaving a wide range of purified proteins such as casein, elastin, bovine serum albumin, and collagen. It cleaves at the C-terminal side of hydrophobic amino acid residues (e.g. Phe, Met, and Ala) (St. Leger *et al.*, 1987a). Pr2 is a trypsin-type enzyme with a specificity for polypeptides containing the basic amino acids Lys and Arg. Pr2 degrades casein and albumin but not elastin (St. Leger *et al.*, 1987a; Cole *et al.*, 1993). Pr4 is a cysteine proteinase. Like Pr2 it attacks substrates on the C-terminal side of Arg and Lys, but is rather more discerning in its requirements, strongly preferring elongated peptide substrates with Arg in the P_1 position (Cole *et al.*, 1993). Pr3 has been less completely studied, but has an acidic pH optimum (St. Leger *et al.*, 1987a). Additional minor acidic proteases occur in some isolates of *M. anisopliae* (St. Leger *et al.*, 1987c). More limited work on other deuteromycete entomopathogens, *B. bassiana*, *Verticillium lecanii*, *Nomuraea rileyi* and *Aschersonia aleyrodis* reveals a similar picture, with each fungus producing multiple cuticle-degrading proteinases in culture (St. Leger *et al.*, 1987c). However, cultures of three *Entomphthorales* spp. led to the production of only single proteinases in each case, which had activity against both trypsin and chymotrypsin substrates (Samuels *et al.*, 1990).

In at least some strains of *M. anisopliae*, Pr1 appears to be important for successful invasion of the host insect by the fungus. The best studied isolate is *M. anisopliae* ME1. Pr1 purified from strain ME1 is able to degrade insect cuticle *in vitro* (St. Leger *et al.*, 1986b), and the enzyme is produced *in vivo* during penetration of the cuticle by the fungus (St. Leger *et al.*, 1989; Goettel *et al.*, 1989). Cuticles of infected insects show clear ultrastructural signs of being eroded by the fungus (Hassan and Charnley, 1989). Most importantly, when turkey egg-white inhibitor (an inhibitor of Pr1) was applied to the cuticle of *Manduca sexta* larvae at the same time as infective conidia of *M. anisopliae* ME1, the inhibitor significantly delayed the mortality of the treated insects (St. Leger *et al.*, 1988a). However, isolates of *M. anisopliae* with proven pathogenicity exist in which little or no Pr1 is produced when grown on insect cuticle *in vitro* (Gupta *et al.*, 1991) so that it is possible that its role can be taken over by other proteinases in some strains. The *M. anisopliae* gene encoding Pr1 has now been cloned (St. Leger *et al.*, 1992), so that it should now be possible to determine the enzyme's role in pathogenesis and virulence by molecular genetic means.

Pr1's specificity is well matched to its job as a cuticle-degrading enzyme. The enzyme's preferred substrate of those tested was Suc-$(Ala)_3$-Phe-NA (Suc = succinimyl; NA = β-naphthylamide), although Suc-$(Ala)_2$-Pro-Phe-NA is also readily accepted. Thus any proteins with sequences containing $(Ala)_n$ repeats will be particularly succeptible to Pr1 attack. This is in fact a characteristic of a number of structural proteins from the cuticle of the locust *Locusta migratoria* (Andersen *et al.*, 1995), with the sequences Ala-Ala-Ala, Ala-Ala-Pro, Ala-Ala-Pro-Ala, and Ala-Ala-Val-Ala particularly well represented. Other insects also have similar sequences in at least some cuticle proteins.

The role of the other *M. anisopliae* proteases in pathogenicity is less clear. Pr2 has been suggested to play a regulatory role in cellular processes within the fungus (St. Leger *et al.*, 1987a; Charnley and St. Leger, 1991), although Pr2 is found on the cuticle during infection (St. Leger *et al.*, 1987b). Pr2 was originally said to be much less effective (only 4–10% as active) in degrading ground locust cuticle than Pr1 (St. Leger *et al.*, 1987a), but in a more recent study (Cole *et al.*, 1993) was found to be rather more effective than previously stated (21% as active as Pr1). Pr4 is produced *in vitro* only after several days in culture, and may function only late in the infection process. Pr4 is a good degrader of cuticle (51% as active as Pr1). The late production of Pr4 seems to rule it out as an agent of cuticle degradation *in vivo*, but it should be borne in mind that the enzyme might be produced earlier during infection than in culture.

Moreover, it is possible that the roles of the various enzymes may differ according to the type of cuticle being attacked. The specificities of the enzymes vary, so that specific and regional differences in cuticle proteins (see Andersen *et al.*, 1995) might be exploited by one enzyme or another. Moreover, since the production of all three enzymes is affected by the nutritional status of the fungus, their production might vary according to the circumstances prevailing during the infection process. For example, the regulation of Pr1 and Pr2 has been studied by St Leger *et al.* (1988b, 1991) and by Paterson *et al.* (1993, 1994a,b). Both enzymes are subject to multiple regulation, including both carbon and nitrogen de-repression, and induction. Under conditions of carbon and nitrogen starvation, Pr2 can be induced by a range of proteinaceous substrates, but Pr1 is induced only by cuticular proteins. It has been suggested that the cuticle-degrading activity of Pr2 may produce soluble peptides that in turn induce Pr1 (Paterson *et al.*, 1994b). Samuels and Paterson (1995) speculated that Pr4 may be produced as specific response to the inhibition of Pr1 and Pr2 by serine protease inhibitors present in insect haemolymph. This might occur only when the infection process was well under way.

Insect pathogenic fungi also produce peptidases. Aminopeptidases are known to be produced by *Entomophthorales* spp. (Samuels *et al.*, 1990), and two enzymes from *M. anisopliae* have been partially characterized (St. Leger

et al., 1993). These last are aminopeptidase and a dipeptidyl peptidase. The aminopeptidase's best substrate was Ala-β-naphthylamide, while the dipeptidyl peptidase showed a strong preference for substrates with a penultimate Pro residue. These enzymes had no detectable activity against insect cuticle when assayed on their own, but as we have seen in the case of the insect moulting fluid peptidase MFP-2, this does not mean that such enzymes can have no role in cuticle degradation. It seems likely that the *M. anisopliae* peptidases act only on the products of the initial attack by proteinases like Pr1. As previously noted, the action of Pr1 on the repetitive motifs common in locust cuticle would provide peptides rich in both Ala and Pro residues. Histochemical evidence is available that aminopeptidase activity is produced *in vivo* during infection (St. Leger *et al.*, 1987b).

Chitinolytic enzymes have been less well studied in entomopathogenic fungi, but sufficient information is available to present an account of the role of these enzymes in cuticle degradation during penetration. Both chitinase and β-N-acetylglucosaminidase are produced in suitable cultures of *M. anisopliae* (St. Leger *et al.*, 1986a), and it might be supposed that these enzymes would play a key role in the fungal attack on the insect's exoskeleton. However, the production of chitinase *in vitro* in cultures of fungus on ground cuticle is late (St. Leger *et al.*, 1986a), and when the production of enzymes was examined during infection of wing cuticles from *Calliphora vomitoria* and larval cuticle from *Manduca sexta*, no evidence for the production of chitinase could be obtained (St. Leger *et al.*, 1987b). While it is possible that some chitinase was present but was strongly absorbed onto endogenous chitin, no GlcNAc was released from cuticle during incubation, and the authors concluded that chitinase production was probably unimportant as an agent of cuticle degradation compared with Pr1 and Pr2. Interestingly, however, β-N-glucosaminidase was found to be present in all cultures.

The key point here is that chitinase (although not β-N-glucosaminidase) is subject both to inductive and repressive controls (St. Leger *et al.*, 1986c). Chitin itself induces synthesis of chitinase, probably through the release of GlcNAc, although glucosamine was also capable of inducing the enzyme. Addition of carbohydrate, lipid or proteins to chitin-grown cultures repressed production of chitinase. By contrast, β-N-acetylglucosaminidase was produced under all conditions. St. Leger *et al.* speculated that while this enzyme may also have other functions in the fungal cell, the continuous production of β-N-acetylglucosaminidase might have the adaptive function of ensuring that the presence of chitin as a feedstock is recognized by the release of low levels of GlcNAc. We might also comment that β-N-acetylglucosaminidase is probably necessary to prevent the inhibition of chitinase by GlcNAc oligomers. This might be important during the process of induction when only low levels of chitinase are available.

Presumably during the process of infection of insect cuticle by *M.*

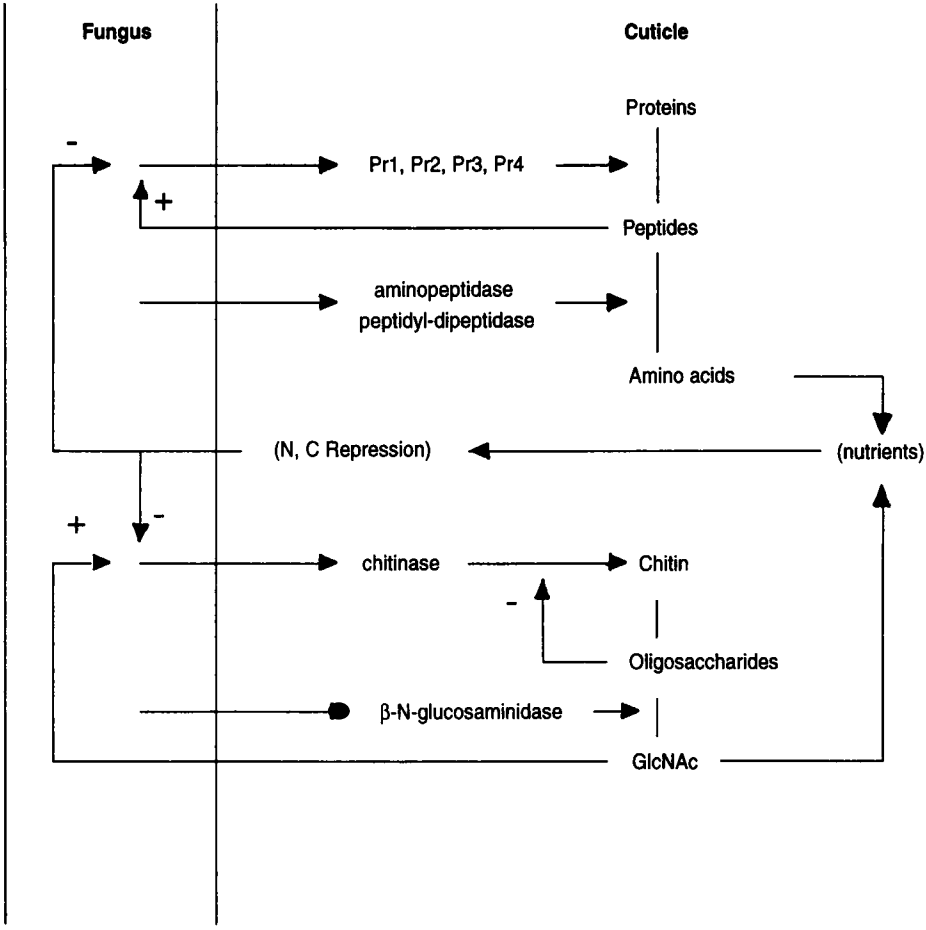

FIG. 10 Cuticle-degrading enzymes of the entomopathogenic fungus, *Metarhizium anisopliae*, and their control. See text for further details.

anisopliae, initially the chitin that is present in the cuticle is largely shielded by the associated cuticular proteins from the attentions of any chitinolytic enzymes that might be present, and is so unavailable to attack. Thus there is no GlcNAc available to induce chitinase production. As the fungus begins to penetrate the cuticle, the secreted proteases Pr1 and Pr2 digest away these proteins rendering chitin available. Low levels of chitinase, combined with constitutively produced β-N-acetylglucosaminidase may now generate small amounts of GlcNAc. However, by this time the specific induction of chitinase by GlcNAc may be overruled by repression due to the general availability of nutrients from other sources. This scenario is illustrated in Fig. 10.

What may be learnt from these fungal proteases systems by the student of insect moulting fluid? The first lesson is that enzymic attack on the cuticle is primarily on cuticle protein. Chitin seems to be relatively unimportant. Second, although the attack on cuticle proteins seems to involve several endo-acting proteolytic enzymes with different specificities (this is true for deuteromycetes, but may not be true of entomophthoralean fungi), there is a single enzyme that seems much more important than others. Third, the production of these enzymes is subject to both inductive and repressive controls.

How does this compare with the situation in insect moulting fluid? As yet we know much less about moulting fluid enzymes than we do about their fungal counterparts. However, it seems to be true for both fungi and moulting insects that the cuticle is initially attacked by proteinases. In pharate adult *Manduca sexta*, the most important enzyme appears to be MFP-1, but it is also evident that the moulting fluid contains many proteolytic enzymes yet to be described. The principal cuticle-degrading proteinase of *M. anisopliae*, Pr1, has a different specificity from that of MFP-1. It seems premature to speculate as to whether the substrate specificities of the two enzymes are adaptive, conferring particular advantages on the fungus and the insect respectively, until more is known about the specificities of enzymes secreted by other fungi and other insects. However, since *M. anisopliae* is a fungus with a wide host range, it seems likely that there is a need for its cuticle-degrading proteinases to be able to attack a wide range of cuticle proteins. The insect enzymes might evolve so as to be much more specific. The production of fungal cuticle-degrading enzymes seems to be regulated at least in part by inductive and repressive controls dependent on the supply of nutrients. The regulation of production of insect moulting fluid enzymes is so far only known to involve control by ecdysteroid moulting hormones. Whether the rise and fall in synthesis of these enzymes is also regulated by metabolic inductive and repressive controls is not yet known.

However, there seem to be some differences between the cuticle-degrading strategies of insects and entomopathogenic fungi. In *M. anisopliae*, it seems as though chitinase is relatively unimportant, being produced relatively late, if at all, during the process of cuticle penetration. By contrast, in insects, chitinases are produced early in the moulting process, and are known to be important (inhibition of chitinase is lethal – see Section 5). This is presumably the consequence of different rationales for the need to digest the cuticle in the two cases. A moulting insect needs to digest the old cuticle primarily to allow it to be shed. This requires the rather complete dissolution of the procuticle, so as to reveal the moulting lines of weakness. As the structural integrity of the cuticle depends on both protein and chitin, it will be necessary to attack both together. Because the period of time spent with a mechanically weakened cuticle must be minimized (the insect must be inactive during this period), it will be important to attack the components of the old cuticle in

a coordinated way. This will require both proteolytic and chitinolytic attack at the same time. As we have discussed (Section 3.5.1), proteinases are likely to initiate cuticle degradation, acting to reveal chitin for the attention of chitinases, but nevertheless both proteinases and chitinases will be needed for the complete dissolution of the old cuticle. Recovery of nutrients from the old cuticle is an important benefit of moulting, but is secondary.

In the case of the fungus, however, the need is to penetrate the cuticle as quickly as possible so as to allow the fungus to establish itself within the insect. If penetration is not speedy, the insect may be able to mount induced immune defences. The complete dissolution of the cuticle is not an objective, since all that is needed is to produce a path of reduced mechanical resistance that can be penetrated by the growing hyphal tip. Electron micrographs of insect cuticle during the process of penetration by the invading fungus show that damage to the cuticle is quite local to the region around the growing hyphae (Hassan and Charnley, 1989). Moreover, the fungus initially lacks its own store of nutrients and must obtain these from its host in order to grow and synthesize new materials. Therefore, the fungus is likely to produce only those enzymes that are necessary to penetrate the cuticle, and to maximize the flow of nutrients from a minimal biosynthetic investment. Proteinases liberate free amino acids from the cuticle, supplying the fungus with both carbon and nitrogen for growth. There would be no particular advantage from the production of chitinolytic enzymes. An additional point to consider is that an insect pathogenic fungus is likely to gain advantage from a cuticle-degrading strategy that maintains the host cuticle's effectiveness as a barrier to infection from other invading micro-organisms as long as possible. This too might argue against the production of chitinases that would compromise the procuticle's mechanical supporting role.

4 Hormones and moulting fluid

4.1 INDUCTION OF CHITINASE BY INCREASED ECDYSTEROID TITRE

The secretion and subsequent activation of chitinolytic enzymes are both clearly regulated by ecdysteroid moulting hormones. The cyclic appearance of the enzyme was first noted to be synchronized with the moulting cycle in *Bombyx mori* by Jeuniaux (1961; 1963), and it is reasonable to suppose that this is driven by the actions of ecdysteroid moulting hormones.

The time of appearance of chitinase coincides with the pre-ecdysial rise in ecdysteroid titre. Kimura (1973) showed that the activity of the *Bombyx* integumental moulting fluid enzymes must be stimulated by injections of ecdysterone (20-hydroxyecdysone; 20-HE) into newly moulted final instar larvae, since these injections provoked not only the synthesis of new cuticle, but also the digestion of the old one. This phenomenon was investigated

further using isolated (ligated) abdomens of fourth (penultimate larval) stage silkworm larvae, about two-thirds of the way through the feeding stage. It was shown that 20-HE injections provoked a fivefold increase in integumental chitinolytic activity (measured as release of GlcNAc from a colloidal chitin substrate). The response was rather sensitive, requiring only 0.5 μg 20-HE, the same dose that was required to induce larval moulting. Although the level of chitinolytic enzymes in uninjected control abdomens declined continuously after ligation, the extent of induction by a supramaximal dose of 20-HE (1.5 μg) remained the same (about fivefold). Interestingly, a basal level of chitinolytic activity was present at all times in the isolated abdominal integument, even though the donor insects were not preparing to moult.

Kimura (1973) claimed that the induction of chitinolytic activity was not a result of *de novo* synthesis of an enzyme ('chitinase'), asserting that injections of actinomycin D and puromycin did not prevent the hormonally induced increase in activity. His conclusion was that specific RNA and protein synthesis were not required for the induction of chitinase, suggesting instead that 20-hydroxyecdysone was involved in the transfer of pre-existing chitinase from the epidermal cells to the endocuticle. Although he did not say so, it is clear from his methods that if this were the case, then the pre-existing chitinase must have been present as an inactive form. This would of course allow passage through newly formed cuticle without chitin digestion occurring, as has been discussed above. However, it is not clear to the present reviewers that Kimura's results really allow these conclusions to be drawn. The doses of actinomycin D and puromycin that he used did in fact depress chitinase expression considerably, much in line with the extent to which the drugs inhibited the incorporation of [^{3}H]-uridine into total RNA, and of [^{14}C]-amino acids into protein.

The problem was revisited by Koga *et al.* (1991), using better enzyme assay procedures and Western blots. It was shown that when *Bombyx* isolated abdomens were given 20-HE injections, low doses of hormone (1.5 $\mu g\,g^{-1}$) induced only β-N-acetylglucosaminidase, while much higher doses (7.5 $\mu g\,g^{-1}$) were needed to induce chitinase. The higher absolute levels of hormone required may have been due to the use of fifth-stage larvae rather than the fourth-stage larvae used by Kimura. A similar result was obtained with fifth-stage *Manduca* larvae by Fukamizo and Kramer (1987). In this insect, injections of modest amounts of 20-HE (1 $\mu g\,g^{-1}$) were found to increase only β-N-acetylglucosaminidase activity in the integument, while much larger quantities of hormone (15 $\mu g\,g^{-1}$) were required to induce chitinase activity. Koga *et al.* (1991) found that the 20-HE treatment in *Bombyx* induced the expression of not only the active 88 kDa and 65 kDa forms of chitinase, but also an inactive 215 kDa form (recognized by its immunoreactivity), supposed by them to be a zymogen. Zymogenic forms of β-N-acetylglucosaminidase were not observed however. In *Manduca* by

contrast (Fukamizo and Kramer, 1987), there was no evidence for the induction by 20-HE of a zymogen of either enzyme.

Interestingly, in both *Manduca* and *Bombyx*, whereas β-N-acetylglucosaminidase was present in the integument of ligated abdomens at all times, chitinase was only detectable following hormone treatment. Considering Kimura's findings with 'chitinase', this suggests that some or all of the activity measured in his paper may really have been β-N-acetylglucosaminidase. As noted by Fukamizo and Kramer (1985b), this enzyme can show GlcNAc-releasing activity depending on the nature of the substrate employed. Kimura's colloidal chitin substrate may have been susceptible to β-N-acetylglucosaminidase attack because it was already partially degraded. Again the finding that β-N-acetylglucosaminidase is present in the integument before any cuticle degradation takes place supports the idea (see above) that the initial enzymic attack on chitin is normally dependent on the action of chitinase.

Kramer *et al.* (1993) have shown that injections of 20-HE into ligated abdomens of *M. sexta* larvae caused a tenfold increase in both epidermal and gut tissues in the amount of mRNA encoding chitinase I. Ligation on day 2 of the fifth stage prevented the appearance of this chitinase mRNA, an event normally seen on days 5–8 of 5th instar larval development. Injection of 20-HE on the second and third days after ligation caused a sharp increase in the amount of chitinase mRNA in epidermal and gut tissues, although induction was greater in the epidermis. Interestingly, chitinase expression rose in the gut 1 day before the epidermis. Kramer *et al.* (1993) suggested that the increase in chitinase mRNA levels was the result of enhanced gene transcription although they did not rule out the possibility that the hormonally induced effects were due to changes in the rates of RNA turnover or processing.

4.2 INDUCTION OF MFP-1 BY FALLING ECDYSTEROID TITRE

Our own work (Samuels and Reynolds, 1993a; 1993b) with pharate adult *Manduca* indicates that in this insect the initiation of the degradation of the old cuticle is controlled largely by a pre-ecdysial decline in ecdysteroid titre which activates the cuticle-degrading proteinase MFP-1.

Chitinolytic enzymes seem not to be involved in this control system. The activity of chitinase does not change significantly during the four days prior to ecdysis, and that of β-N-acetylglucosaminidase changes only slightly, reaching a slight but significant peak well before the moulting fluid acquires its maximum cuticle-degrading activity (see above, Section 3.4 – Samuels and Reynolds, 1993a). Furthermore, injection of 20-HE (25 μg) does not significantly affect moulting fluid chitinase activity, and has only a marginal effect on β-N-acetylglucosaminidase activity (Samuels and Reynolds, 1993b).

These findings do not conflict with the work reviewed above which reports the induction of both chitinase and β-N-acetylglucosaminidase by 20-HE. These studies all concerned insects that had not yet initiated moulting, and found that an increase in 20-HE titre resulted in an increase in the activities of the two enzymes. By contrast, in our work we examined the effect of 20-HE on insects in which moulting was well under way, and in which these enzymes were already being expressed at a high level. However, since the hormonal treatment that we used was sufficient to delay both eclosion and the thinning of the pupal cuticle, it follows that the initiation of cuticle degradation is independent of changes in either of these enzymes.

The picture for moulting fluid proteases is quite different. The increase in activity of MFP-1 and MFP-2 prior to the adult eclosion of *Manduca* (Fig. 9 – Samuels and Reynolds, 1993a) is the inverse of the declining ecdysteroid titre at this time (Bollenbacher *et al.*, 1981). It has been found (Schwartz and Truman, 1983) that injection or infusion of 20-HE at this time delays eclosion, and the associated developmental events that precede it, including the digestion of the old cuticle and the resorption of the moulting fluid. We have shown (Samuels and Reynolds, 1993b) that injections of 20-HE given about 48 h prior to the expected time of eclosion inhibit the normal pre-eclosion rise in the cuticle-degrading ability of the moulting fluid. The effect is dose dependent in the range 5–25 μg, and closely parallels the effects of these injections on the thinning of the cuticle. A dose of 25 μg 20-HE reduces the ability of moulting fluid taken 24 h later to release [^{3}H]-labelled amino acids from biosynthetically labelled cuticle by more than 90%. This effect is accompanied by a similar reduction in the activity of MFP-1 in the moulting fluid. The effect is specific to MFP-1. The activity in moulting fluid of the aminopeptidase MFP-2 did not change significantly after 20-HE treatment. It is not clear what controls the activity of this enzyme (which increases in activity considerably during the four days preceding eclosion) under normal circumstances.

The situation is illustrated in Fig. 11.

4.3 JUVENILE HORMONE

The effects of juvenile hormone (JH) on moulting fluid enzyme production have not been extensively investigated. Since a moult from larva to pupa, or pupa to adult, will require the degradation of the old cuticle just as much as those from larva to larva, it would be expected that JH's effects would be related largely to its modulatory effects on ecdysteroid action (Riddiford, 1985).

Kimura (1973) investigated the effects of 20-HE injections on isolated *Bombyx* abdomens. As detailed above (Section 4.1.1) he found that these injections caused the initiation of a larval–larval moult, and also caused an increase in integumental chitinolytic enzyme activity. He varied the treatment

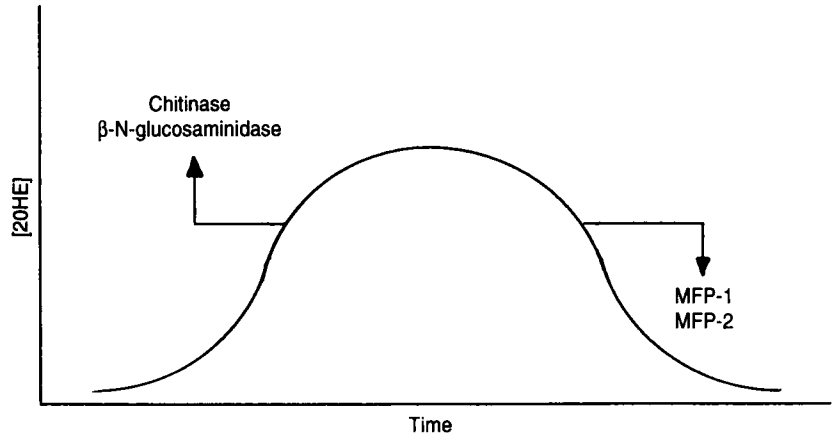

FIG. 11 Control of moulting fluid enzyme activity by ecdysteroid moulting hormones. See text for further details.

given by changing the length of time that elapsed between ligation and subsequent 20-HE treatment. The longer the period that elapsed, the more likely was the abdomen to respond to the hormone injection by the production of a pupal cuticle, instead of a larval one. This was explained as being a consequence of the decline in the JH titre within the ligated abdomen. Interestingly, the relative increase in chitinolytic activity produced in response to a standard dose of 20-HE was not affected by the type of new cuticle that was produced. This result implies that JH does not directly affect the expression of the enzyme that was assayed (as explained in Section 4.1.1, this may not have been chitinase, as claimed in Kimura's paper, but β-N-acetylglucosaminidase).

In apparent contrast to this, Kramer *et al.* (1993) found that topical application of a juvenile hormone mimic, fenoxycarb, could prevent the increase in the expression of a chitinase gene caused by injecting 20-HE into isolated abdomens of fifth instar *Manduca* larvae. Fenoxycarb abolished the 20-HE-induced increase in the gut entirely, and in the epidermis almost entirely. Fenoxycarb alone had no effect on gut chitinase mRNA levels, although the fenoxycarb-treated abdomens showed a slight increase in integumental chitinase over solvent-treated controls. Unfortunately, Kramer *et al.* (1993) did not report whether the fenoxycarb treatment also prevented the induction by 20-HE of apolysis and new cuticle formation. The experience of the present reviewers is that it would have done so. If this was the case, the effect of the JH mimic would have been to suppress 20-HE-induced moulting, and the inhibition of chitinase gene expression by JH should be seen in this light, rather than a specific effect on chitinase expression *per se*.

5 Insecticides and moulting fluid

Very few investigators have explored the possible effects of insecticides on moulting fluid function. However, a number of studies have explored the potentially lethal effects on insects of interfering with cuticle degradation.

An insecticide might interfere with normal moulting by altering the expression of an endogenous cuticle-degrading enzyme. Acylurea insecticides such as diflubenzuron kill insects by interfering with the deposition of chitin in the cuticle. The affected insects die at the next moult because the new cuticle is too weak to support the strain of ecdysis. It is now known that these compounds interfere rather selectively with chitin synthesis, although the precise manner in which they inhibit chitin synthetase is not understood. Almost certainly the action is not direct upon the synthetase, since broken-cell extracts synthesizing chitin are not inhibited by diflubenzuron (reviewed by Reynolds, 1987). An early hypothesis was that the insecticide might act to stimulate the production of chitinolytic enzymes in the affected integument. Ishaaya and Casida (1974) measured the level of chitinase in housefly (*Musca domestica*) larvae fed diflubenzuron-containing diets. They found that increasing levels of the pesticide were associated with increased levels of chitinase in affected larvae. It is now generally accepted, however, that these changes are not directly related to the insecticidal action of diflubenzuron, but are more likely to be an indirect consequence of the disruption that the pesticide causes to the insect's growth and moulting cycles.

On the other hand, Koga *et al.* (1991) found that treatment of *Bombyx* larvae with the candidate imidazole insecticide KK-42 (Kuwano *et al.*, 1985) suppressed both the larval-pupal transformation, and prevented the expression of both chitinase and β-N-acetylglucosaminidase. This compound has insecticidal activity of the insect growth regulator (IGR) type, and has been variously suggested to have anti-JH activity (Kuwano *et al.*, 1985), and to be an inhibitor of ecdysteroid synthesis (Kadono-Okuda *et al.*, 1987). Because it is not yet clear how this compound acts, it is not possible to interpret this finding in terms of hormonal control of chitinolytic enzyme expression in *Bombyx*.

Another way in which insect pests might be controlled through the moulting system would be to expose them to exogenous cuticle-degrading enzymes. It is unlikely that these would do much damage to the insect from the outside, but the insect's peritrophic membrane might be more vulnerable. This has been seriously considered as a way of enhancing the efficiency of microbial insecticides. Smirnoff (1974) and Morris (1976) reported that when *Bacillus thuringiensis* was applied together with bacterial chitinase to the spruce budworm larvae (*Choristoneura fumiferana*), the insects died more quickly; Shapiro *et al.* (1987) found the same was true when gypsy moth larvae (*Lymantria dispar*) were exposed to a baculovirus at the same time

as chitinase. Presumably the insect's peritrophic membrane poses a barrier to infection (Spence, 1991), and the exogenous chitinase may erode this. In fact, AcMNPV produces its own chitinase (Gopalakrishnan *et al.*, 1995), and it is possible that insect pathogenic bacteria (Brandt *et al.*, 1978) and viruses (Stolz and Summers, 1971) may use enzymes to penetrate the peritrophic membrane barrier.

A more promising approach would be to use micro-organisms to cause pest insects to express their own (or similar) moulting fluid enzymes in the wrong place and/or at the wrong time. Gopalakrishnan *et al.* (1995) constructed a baculovirus (AcMNPV) with a *Manduca sexta* chitinase gene under the control of the virus polyhedrin promoter. This recombinant virus was able to direct the expression of chitinase in insect cells (see Section 3.1.2) and caused the accumulation of chitinase in haemolymph when injected into *M. sexta* and *Spodoptera frugiperda* larvae. Mortality of the infected *S. frugiperda* larvae was enhanced, so that only about 75 h was required for 100% mortality with the recombinant AcMNPV compared with about 100 h in the case of the unaltered virus. It was not determined why the affected insects died. This result suggests that the expression of chitinase by a recombinant insect virus might be a useful way of improving its virulence. The same might be true of other moulting fluid enzymes. As these enzymes are unlikely to be toxic to non-target organisms or to be environmentally damaging, this could be a promising approach to the improvement of microbial pesticides.

An approach more familiar to the agrochemical industry would be to kill moulting insects by inhibiting endogenous moulting fluid enzymes with chemicals. Bade and Shoukimas (1974), having discovered the presence in the moulting fluid of *Manduca sexta* larvae of a neutral, metal chelator-sensitive protease (almost certainly the same as the MFP-2 of Samuels *et al.*, 1993b), reasoned that it might be possible to adversely affect moulting of that insect by giving food containing a metal chelator. They found that feeding 1,10-phenanthroline was lethal to *M. sexta* larvae: 0.02% in artificial diet arrested all the larvae given this food at the third stage, while 0.06% was lethal in the first stage. This would be more impressive if it had been definitely shown that death was due to failed moulting. However, even this would not necessarily prove that the chemical had its effect specifically on moulting, because it is the routine experience of all who keep caterpillar cultures that these insects almost invariably die at the moult when they are unwell, whatever the cause. In fact, the matter was later resolved when Rebeiz *et al.* (1990) showed that 1,10-phenanthroline kills *Trichoplusia ni* larvae by quite a different mechanism involving the inhibition of the porphyrin-haem biosynthetic pathway, and causing the accumulation of massive amounts of protoporphyrin IX. This leads to the death of the insect through the light-induced production of singlet oxygen and perhaps other radicals such as O_2^- and ^-OH.

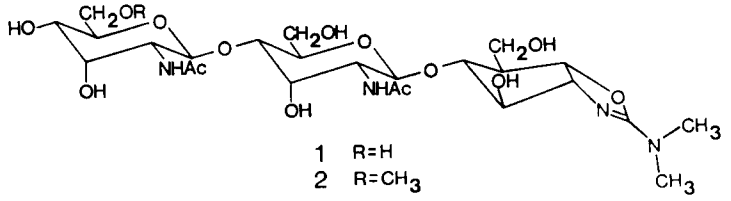

FIG. 12 Structures of allosamidin (**1**) and methylallosamidin (**2**). From Sakuda *et al.* (1987).

As far as we are aware, there are no published reports of the use of proteinase inhibitors to kill insects by interfering with moulting. Where proteinase inhibitors have been used to kill insects (e.g. Hilder *et al.*, 1987), the lethal effects have probably been largely the effect of interfering with digestive function (Broadway and Duffey, 1986). However, the inhibition of chitinase seems as though it may hold some promise as a method of insect control.

A specific inhibitor of chitinase, allosamidin (Fig. 12), has been isolated from cultures of *Streptomyces* spp. It shows insecticidal activity by preventing ecdysis (Sakuda *et al.*, 1987a; 1987b). Independently, Somers *et al.* (1987) discovered the same metabolite, which they named A82516, from another *Streptomyces* isolate. The initial screen was against *Streptomyces griseus* chitinase, but the compound was found to be insecticidal against *Musca domestica* larvae.

Allosamidin is a potent and selective competitive inhibitor of insect chitinases (Koga *et al.*, 1987), inhibiting *Bombyx* integumental and gut chitinases with a K_i of about 0.1 μM, and has very similar activity against integumental chitinase (Koga *et al.*, 1987). In another study, using synthetic allosamidin, Spindler and Spindler-Barth (1994) found that chitinase from a dipteran (*Chironomus tentans*) cell line was inhibited at submicromolar levels ($K_i = 0.5$ μM) while chitinase from the brine shrimp *Artemia salina* was slightly less sensitive ($K_i = 1.6$ μM). The synthetic diastereomer isoallosamidin was much less active in both cases, but the order of preference was the same, with $K_i = 380$ μM for the *Chironomus* chitinase, and $K_i = 2.5$ mM for the brine shrimp enzyme.

A highly significant finding from the practical point of view, is that the inhibitory activity of the allosamidin compounds towards chitinases from non-arthropod sources is much less than towards arthropods. Koga *et al.* (1987) found that allosamidin had no inhibitory activity against a plant chitinase (from yam). This is significant in that plant chitinases are thought to be used by plants as defences against pathogenic fungi (Roberts and Selitrennikoff, 1988), and therefore allosamidin should not be detrimental to plants when

used for crop protection purposes. Koga *et al.* (1987) also reported that inhibitory activity against chitinase from *Streptomyces griseus* and *Serratia marcescens* was only about 0.2% of that against the *Bombyx* enzyme, while Spindler and Spindler-Barth (1994) found that the activity of allosamidin against the chitinase of *Streptomyces griseus* had a K_i of 21 μM, and isoallosamidin was unable to inhibit the microbial enzyme at all. Recently, however, Gooday *et al.* (1988) found that allosamidin was remarkably potent against chitinase activity from the nematode *Onchocerca gibsoni*, giving 50% inhibition at only 0.20 nM. Allosamidin had no inhibitory activity when tested against chicken or human lysozyme, nor against insect β-N-acetylglucosaminidases. Thus, with the one exception of nematodes, the documented actions of allosamidin are highly specific towards arthropod, especially insect, chitinases. Allosamidin and its congeners may well be the prototypes of synthetic insecticidal compounds with highly favourable environmental qualities.

6 Conclusions

Insect moulting has been intensively studied over the last 50 years. Most of the attention that has focused on the developmental programme of moulting has been directed towards the synthesis of the new cuticle. Insect physiologists and biochemists have learnt that this programme is initiated and controlled by hormones (Riddiford, 1991), and agricultural chemists and applied entomologists have learnt that the programme can be disrupted by chemicals that interfere with hormone action, or which interfere with the synthesis of the new cuticle (Menn *et al.*, 1989; Reynolds, 1989). The degradation of the old cuticle by moulting fluid is an essential part of the moulting process that has been much less intensively studied. It is the hope of the authors that this review will help to stimulate interest in moulting fluid. The processes of secretion, activation, enzymic action, and resorption of insect moulting fluid all have parallels elsewhere in animal physiology and biochemistry. We would like to point out, for example, that the action of the moulting fluid in degrading an extracellular matrix might be a useful model for connective tissue remodelling, and some types of inflammation in mammals. The simple spatial arrangement of the cells that secrete the moulting fluid, the fluid itself, and the matrix that is attacked might be particularly helpful in this regard. The accessibility of moulting fluid for sampling; the predictable sequence of moulting fluid secretion, activation and resorption; and its regulation by hormones may also offer an unparalleled experimental opportunity for the student of connective tissue degradation. Moreover, since disruption of moulting is a proven method of killing insects, more attention devoted to the study of moulting fluid physiology and biochemistry might lead to the discovery of novel safer methods of insect pest control.

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Interactions of Cytoplasmic Polyhedrosis Viruses with Insects

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1 Introduction

Cytoplasmic Polyhedrosis Viruses (CPVs) are very common widespread pathogens of insects of economic importance (Smith and Rivers, 1956; Aruga, 1971; Mery and Dulmage, 1975; Belloncik, 1984) or collected from the wild (Hukuhara and Bonami, 1992). Ishimori (1934) was the first to diagnose CPV infection in a *Bombyx mori* larva colony by the abnormal presence of dense inclusion bodies in the insect midgut. Later on, Smith and Wyckoff (1950) demonstrated that these inclusion bodies were related to a disease different than a well-known nuclear polyhedrosis virus infection.

CPVs, isolated from more than 250 species of insects which include 201 Lepidoptera, 39 Diptera, 7 Hymenoptera, and 2 Coleoptera (Hukuhara and Bonami, 1992), are composed of double-stranded RNA (ds RNA). They are classified, with other vertebrate, plant and non-occluded insect viruses, in the cypovirus group inside the Reoviridae family (Holmes, 1991). Along with Nuclear Polyhedrosis Virus (NPV) and Granulosis Virus (GV), CPV infection is characterized by the synthesis in insect cells of an excessive viral coded protein (polyhedrin) which crystallizes including in this process viral particles. These inclusion bodies named polyhedra are responsible for the environmental protection of virions and provide a safe vehicle, in nature, for the passage of viruses from infected insects to others. However, CPVs differ from the two other occluded insect viruses by their intracytoplasmic localization, with some exceptions, of polyhedra, structure and composition of virions, as well as by their biological effects on insect cells *in vivo* or cultivated *in vitro*.

Since these viruses were first demonstrated as economically important pathogens in the silkworm industry, most research has been concentrated, mainly in Japan, towards the prevention of the infection of these insects by CPV. The early studies on viral pathogenesis, biochemical characterizations and replication were conducted almost exclusively on CPV strains of silkworm, and much important fundamental data in virology and in molecular and cell biology, such as the discovery of the CAP structure of messenger RNA (Furuichi and Miura, 1975), were obtained from research on *Bm* CPV. Later on, the pathogenesis of several CPVs of other insects was studied, and the greater importance of these viruses in biological control of insect pests was recognized (Grison *et al.*, 1959; Granados, 1978; Aizawa, 1976; Payne, 1981; Katagiri, 1981; Belloncik, 1989; Chen, 1990). Moreover, development of efficient cell culture systems supporting the replication of these viruses as well as several genetic engineering and molecular biology techniques contributed greatly to the comprehension of some important mechanisms of CPV infection both *in vivo* and *in vitro*.

Several review papers (Kawase, 1971; Payne, 1981; Payne and Mertens, 1983; Hukuhara, 1985; Belloncik, 1989; Hukuhara and Bonami, 1992) have already treated in detail the structure and biochemical properties of CPVs.

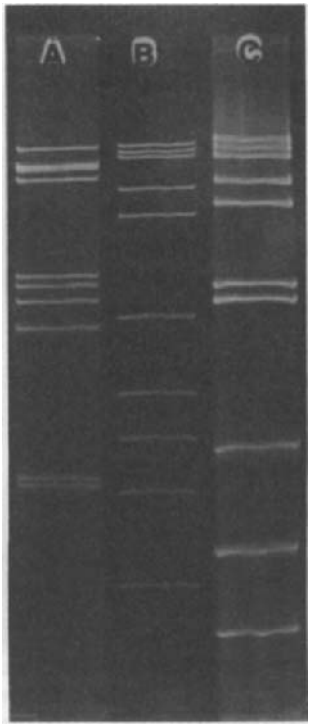

FIG. 1 Electrophoretic pattern ds RNA of Cytoplasmic Polyhedrosis Viruses. (A) *Cf* (undetermined type). (B) *Bm* (type 1), and (C) *Es* (type 5) CPVs. Migration in 7.5% polyacrilamide gel.

Therefore, in this chapter, I will rather review the characteristics of CPV composition and replication in relation to the specificity and persistence of viral infection, together with the influences of the cell for a complete and normal replication and morphogenesis of the viruses. Furthermore, I will illustrate the peculiar interactions of cytoplasmic polyhedrosis viruses with insect cells both *in vivo* and cultivated *in vitro*, together with the different endogenous and exogenous factors that influence the evolution of the viral infection.

2 Main characteristics of CPVs

Cytoplasmic polyhedrosis viruses, genus cypovirus (Holmes, 1991), share with other viruses of the *Reoviridae* family some characteristics such as the virus shape and the presence of a segmented double-stranded RNA genome (Fig. 1). However, virus particle structure differs from vertebrate, plant and

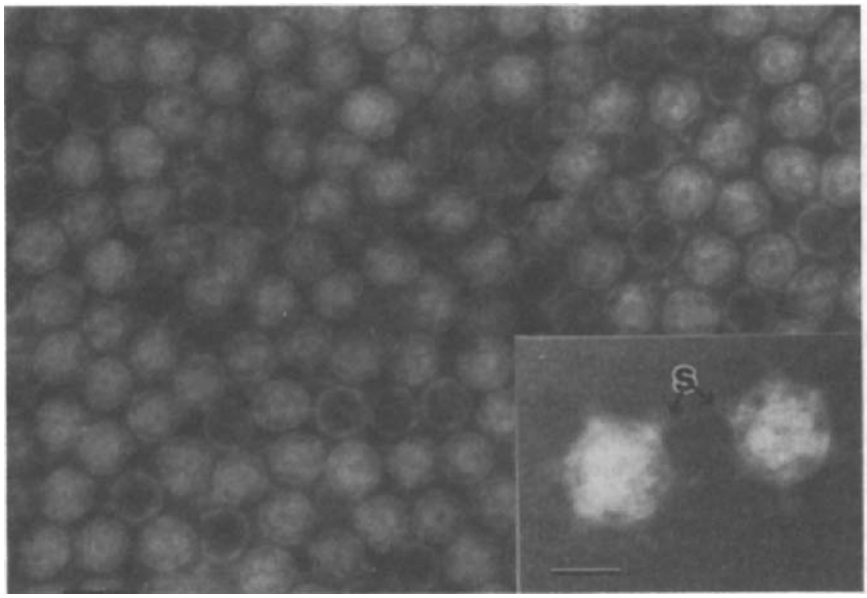

FIG. 2 Suspension of CPV particles. Negative staining. S: spike. Arrow head: Empty particles. Bar = 100 nm (insert, 50 nm). From Dr Claude Lavallée, with permission.

insect reoviruses by the absence of an additional capsid (Lewandowski and Traynor, 1972; Payne and Tinsley, 1974) and hypersynthesis of a viral coded protein (polyhedrin) by infected insect cells. According to some speculations on the evolution of viruses in nature, the second reovirus capsid could be a residue of CPV polyhedrin following their passages in different vertebrate hosts or the polyhedrin could be equivalent to the second capsid of reovirus particles.

The icosahedral virus particle of approximately 70 nm in size has 12 hollowed spikes, one at each verticle of the icosahedra (Fig. 2). It is composed of 3 to 5 polypeptides, 10 segments of ds RNA representing 10 individual genes and enzymes such as transcriptase (Lewandowski *et al.*, 1969), methylase, transferase, and nucleotide phosphohydrolase, which play a crucial role in mRNA synthesis (Storer *et al.*, 1974; Furuichi, 1974, 1978; Shimotohno and Miura, 1977; Mertens and Payne, 1978).

Classification of CPV isolates has been based up to now on their differential electrophoretic profile after migration on a gel of the 10 viral ds RNA segments (Payne and Rivers, 1976). According to this classification, twelve types of CPVs were first designed (Payne and Rivers, 1976; Payne *et al.*, 1977) and two additional types were further recorded (Fouillard and Morel, 1994; Belloncik *et al.*, 1994a). Variations in polyacrylamide concentrations of

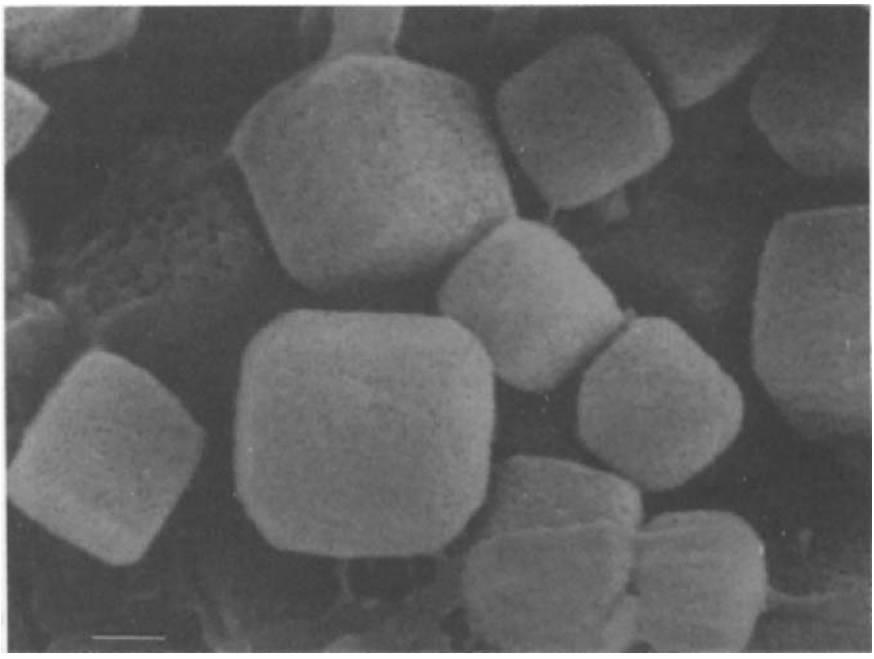

FIG. 3 *Bm* CPV polyhedra. Scanning electron microscopy. Bar = 800 nm. From Dr Claude Lavallée, with permission.

migration gels permit intra-type virus differentiation (Mertens *et al.*, 1989). No RNA homology and/or antigenic relationship has been demonstrated between viruses belonging to distinct types (Payne *et al.*, 1978, 1986; Galinski *et al.*, 1983; Belloncik, 1989; Mertens *et al.*, 1989).

In vitro translation studies on RNA conducted in the rabbit reticulocyte system (McCrae and Mertens, 1983; Hashimoto *et al.*, 1986; Liu *et al.*, 1989) indicated that polyhedrin, which is the major viral protein, is coded by the 10th gene, which is the smallest. The nucleotide sequence of this gene is the only one known (Arella *et al.*, 1988; Fossiez *et al.*, 1989; Liu *et al.*, 1989; Mori *et al.*, 1989). In fact, only the role of this gene is known and the importance of the remaining 9 genes in viral replication and interactions with insect cells merits more intensive study.

The polyhedrin has a molecular weight ranging from 27 000 to 31 000 and crystallizes normally in the cytoplasm in a cubic lattice system called polyhedra. The polyhedra (Figs 3 and 4) are considered as a safe vehicle for the virions in a hostile environment and may contain one (Clark *et al.*, 1969), a few (Fig. 5) or a high number of viral particles (Fig. 6) even reaching more than 10 000 (Arnott *et al.*, 1968; Kobayashi, 1971). An alkaline protease is

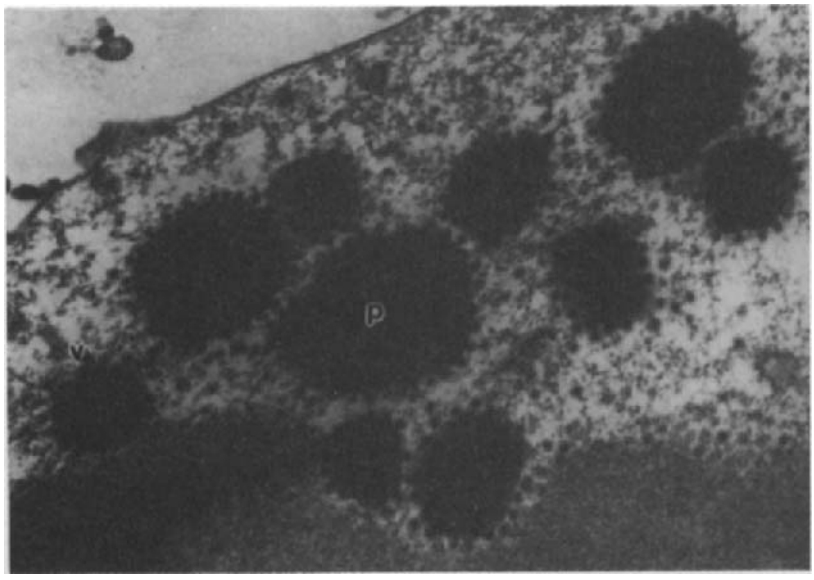

FIG. 4 CPV polyhedra in cytoplasm of infected cells. *Bm* CPV infection of silkworm cell line. Electron microscopy. P = polyhedra. V = Virus particles. Bar = 400 nm.

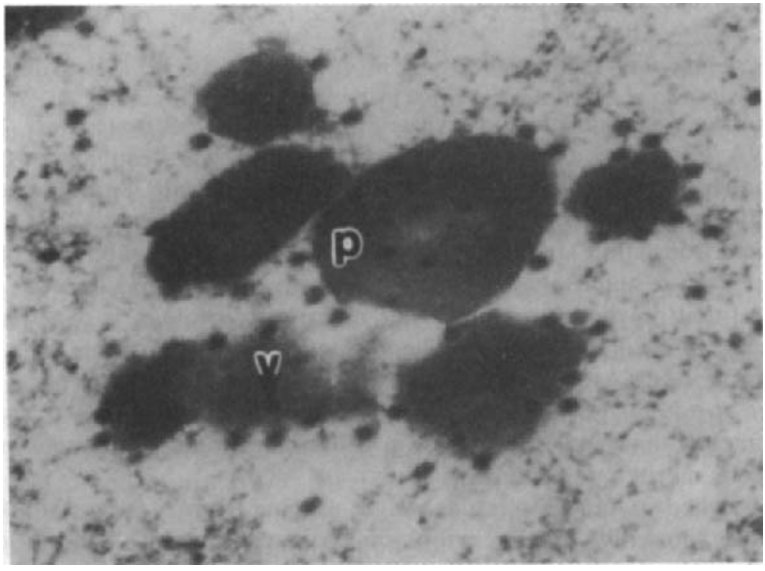

FIG. 5 CPV polyhedra in cytoplasm of infected cells. *Ha* CPV in *H. armigera* midgut. Electron microscopy. P = polyhedra. V = Virus particles. Bar = 250 nm.

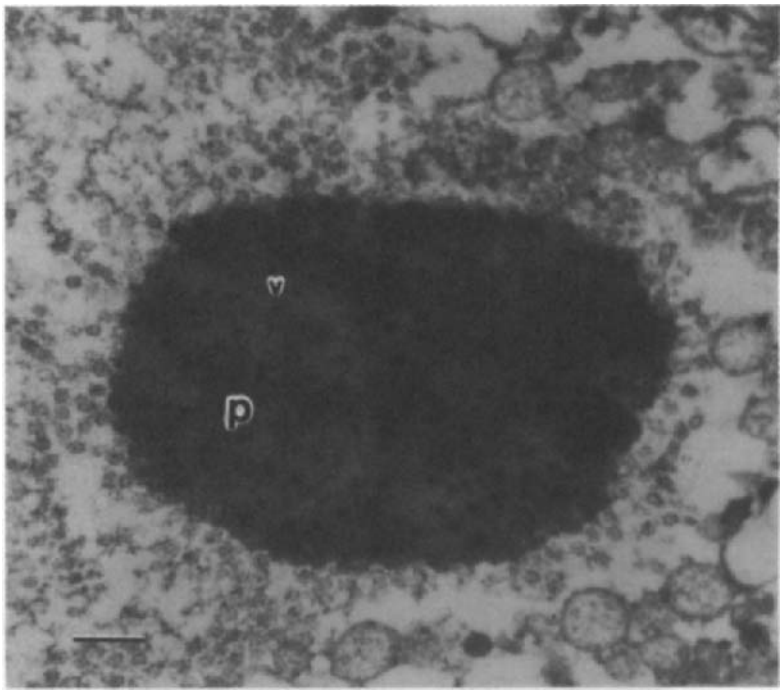

FIG. 6 CPV polyhedra in cytoplasm of infected cells. *Bm* CPV infection of silkworm cell line. Electron microscopy. P = polyhedra. V = Virus particles. Bar = 200 nm.

associated with polyhedra derived from insects (Mori and Kawase, 1983) but not with those obtained from infected cell cultures (Mori *et al.*, 1985). The exact mechanism of polyhedra formation is unknown but it has been demonstrated that interactions, explained later in this chapter, between the virus strain and the cell, govern the process of crystallization as well as the shape and location of polyhedra.

3 Virus ecology

Cytoplasmic polyhedrosis viruses are widely distributed in nature and have been isolated from insects collected in various geographical areas as well as from different climatic and ecological conditions in the world. The host range of CPVs is wide (Katagiri, 1981; Payne, 1981; Belloncik, 1989). However, no CPV infection, in nature, has been conclusively recorded from invertebrates other than insects and the crustacean *Simocephalus expinosus* (Federici and Hazard, 1975) nor from other animal, plant or procaryote organisms.

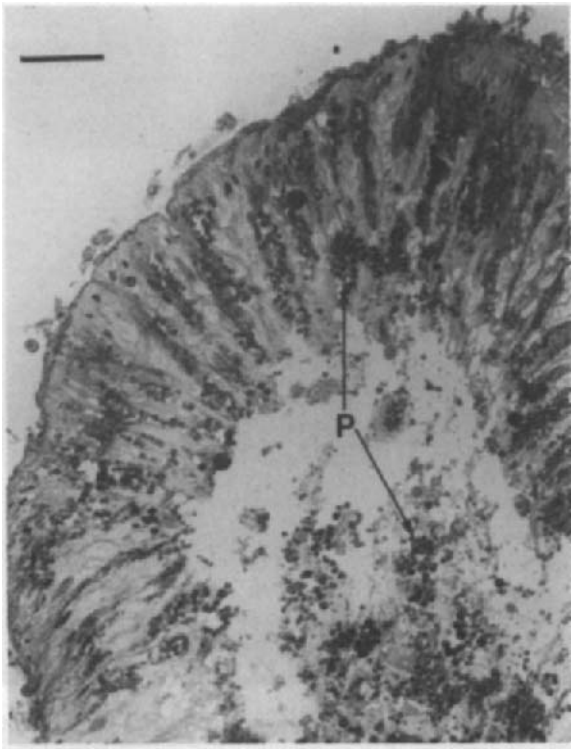

FIG. 7 Infected *E. scandens* midgut with *Es* CPV. Polyhedra in lumen will be excreted in faeces. P: Polyhedra. Light microscopy. Bar = 50 nm.

In nature, polyhedra which contribute to the protection of the virions are released in faeces (Figs 7 and 8) or cadavers of infected larvae. These viral inclusion bodies contaminate the soil (Hukuhara, 1972) and plants, and thereafter are ingested by phytophagous insects. Another mode of transmission of virus infection in nature is by spoilage of the egg surface laid by contaminated females (Sikorowski *et al.*, 1973; Bellemare and Belloncik, 1981). Occasionally, birds, wind, running water, parasites or other means of transportation will disseminate the CPV polyhedra.

Vertical transmission of the virus through the eggs combined to the latency of CPV in insects is suspected to contribute also to the transmission from one generation to another as well as to the persistence of CPV infection (Aruga, 1971; Payne, 1981; Belloncik *et al.*, 1992). However, the occurrence and the importance of these phenomena must be more carefully documented before a final conclusion can be drawn.

Study of CPV ecology is complicated owing to the viral host range as well

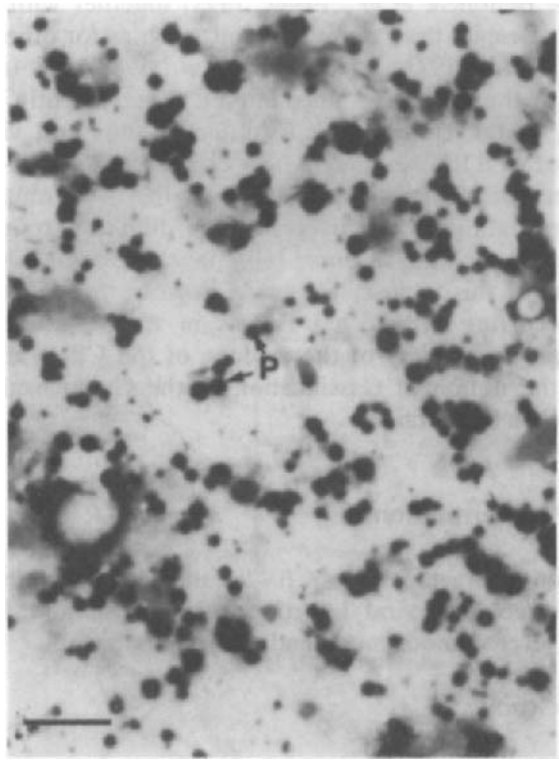

FIG. 8 Smear from infected *E. scandens* midgut with *Es* CPV. P: Polyhedra. Light microscopy. Buffalo-black staining (Sikorowski *et al.*, 1971b). Bar = 20 nm.

as to the viral genome structure and instability. Numerous data collected in nature and laboratory demonstrated that a CPV originated from one insect replicates in other insect species (review of Tanaka, 1971; Yamaguchi, 1979; Belloncik *et al.*, 1980, 1984a; Payne and Mertens, 1983). One particular type or strain of CPV may be therefore propagated, in nature, by several hosts. During this cross-transmission in insects or cell cultures, some CPVs demonstrate stability in their ds RNA electrophoretic pattern (Belloncik, 1989; Belloncik *et al.*, 1989). On another hand predictable (Rubinstein and Harley, 1978) and also arbitrary genomic deletions (Tao *et al.*, 1992; Belloncik *et al.*, 1994a) as well the appearance of a viable dwarf gene (Arella *et al.*, 1988) are noted after passages in the insect of some other CPVs.

Mixed infections of larvae with several CPV types or strains in nature (Payne, 1976; Belloncik *et al.*, 1994a; Tao *et al.*, 1992) can be demonstrated by resolution on gel electrophoresis of more than 10 bands of ds RNA extracted from infected insects. Moreover, infection of an insect with an exogenous CPV could activate replication of an endogenous CPV strain

(Tanaka, 1971; Belloncik *et al.*, 1984a, 1992) together with the exogenous CPV infection. Despite that, no reassortant, such as for other segmented ds RNA viruses (Fields, 1973, 1981; Ward *et al.*, 1988) was isolated. This is probably due to the difficulties encountered, until recently (Belloncik and Arella, 1988), in the cloning of CPV-infected cells and virions. Genetic re-assortment of viral RNA segments between CPVs in mixed-infected insects is not, however, excluded. This mechanism, rather than mutation, could be a plausible explanation for the occurrence of different strains of a CPV of one insect such as those of *B. mori* (Hukuhara, 1985).

In conclusion of this section, we may point out that all the viral genetic phenomena occurring in infected insects in nature as described above contribute to the complexity of the ecology of the CPVs and add another dimension to the notion of classification of the Cytoplasmic Polyhedrosis Viruses into types and strains.

4 Insect–CPV interrelations

The most obvious characteristics of CPV are the highly complex interrelations between the insect and viral infection (Figs 9, 10 and 11). These are influenced by several endogenous and exogenous factors.

4.1 CPV REPLICATION

For a detailed description of the molecular and biological aspects of CPV replication, the reader may refer to the review of Payne (1981).

At an early stage of replication, transcriptase, not sensitive to actinomycin D and present in the virion, acts on the ds viral RNA without proteolytic or heat treatment of the virus particle. *In vitro* experiments (Shimotohno and Miura, 1974) and *in vivo* infections (Furusawa and Kawase, 1971, 1973; Payne and Kalmakoff, 1973) demonstrated the production of ssRNA transcripts of each of the 10 ds RNA segments of the viral genome. The mRNA is synthesized in the virus particle following repeating passages of the circular ds RNA genome through the basal part of the virus where the enzyme complex needed for this synthesis is located, and completed mRNA is excreted from the virions through the virus projections (Yazaki and Miura, 1980; Yazaki and Sano, 1986). Control of the quantity of different polypeptides could be achieved by differences in transcription rates of each segment; the smallest segments are transcribed more than the largest one (Mertens, 1979). The transcription is stimulated by a methyl group donor-S-adenosylmethionine (SAM) and mRNA of the virus possesses at the 5′ terminus a methylated nucleotide sequence or 'cap') (Furuichi and Miura, 1975) which is a crucial component in the initiation of protein synthesis (Mertens, 1979; Shimotohno *et al.*, 1977). It is important to point out that

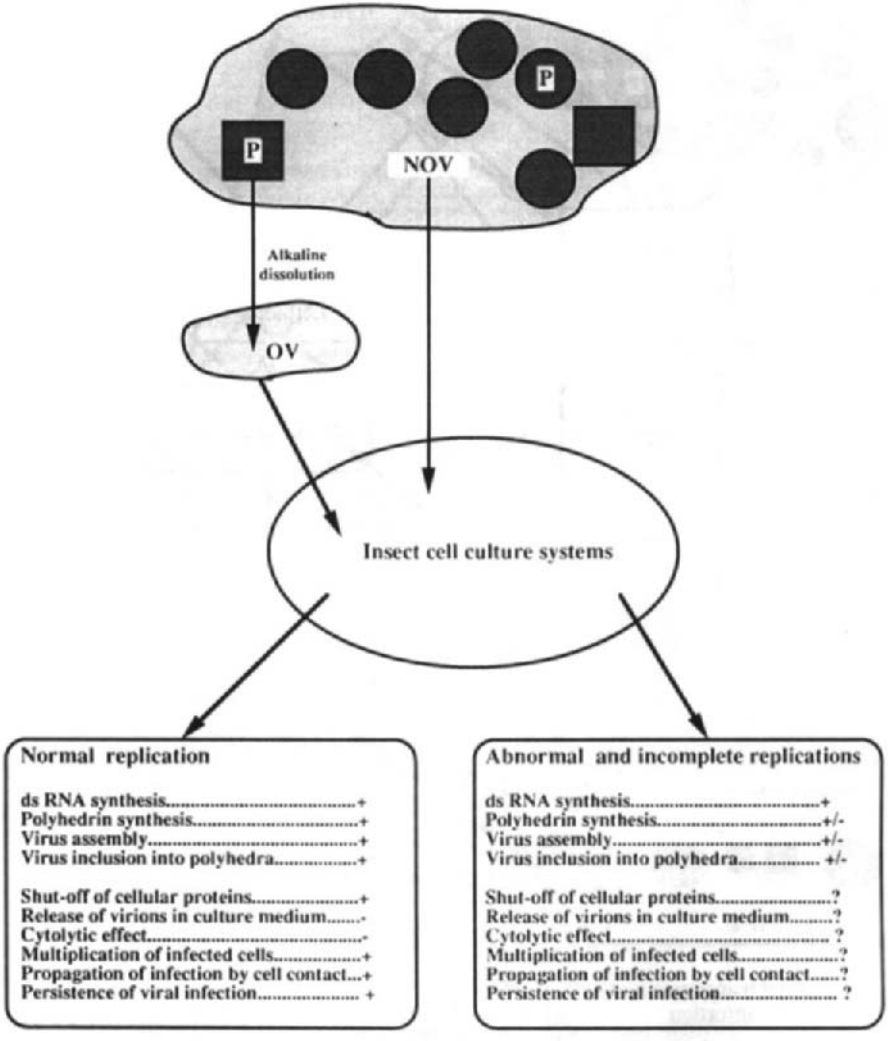

FIG. 9 Comparative patterns of CPV replication in cell culture systems. NOV = Non-occluded virions. OV = Occluded virions. P = Polyhedra.

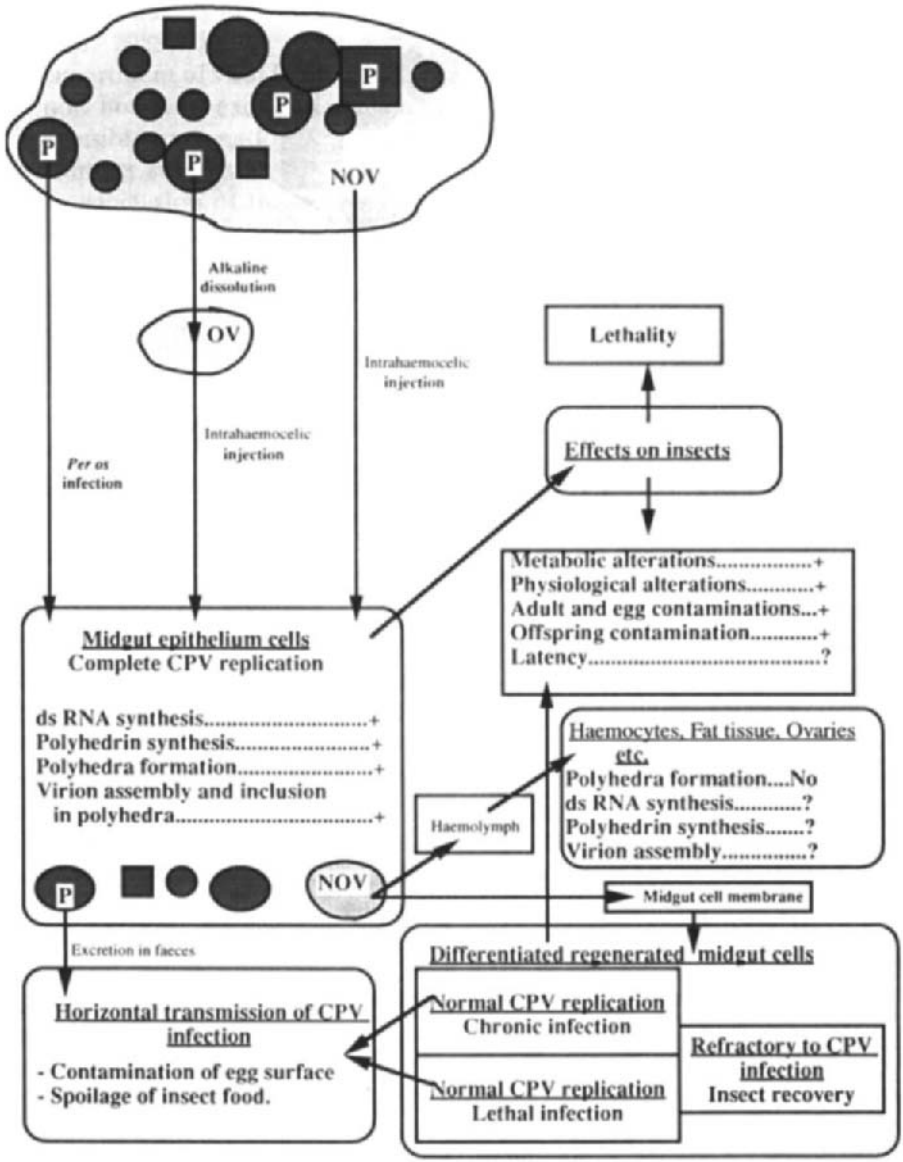

FIG. 10 Schematic representation of CPV infection and pathogenesis in insect larvae. NOV = Non-occluded virions. OV = Occluded virions. P = Polyhedra.

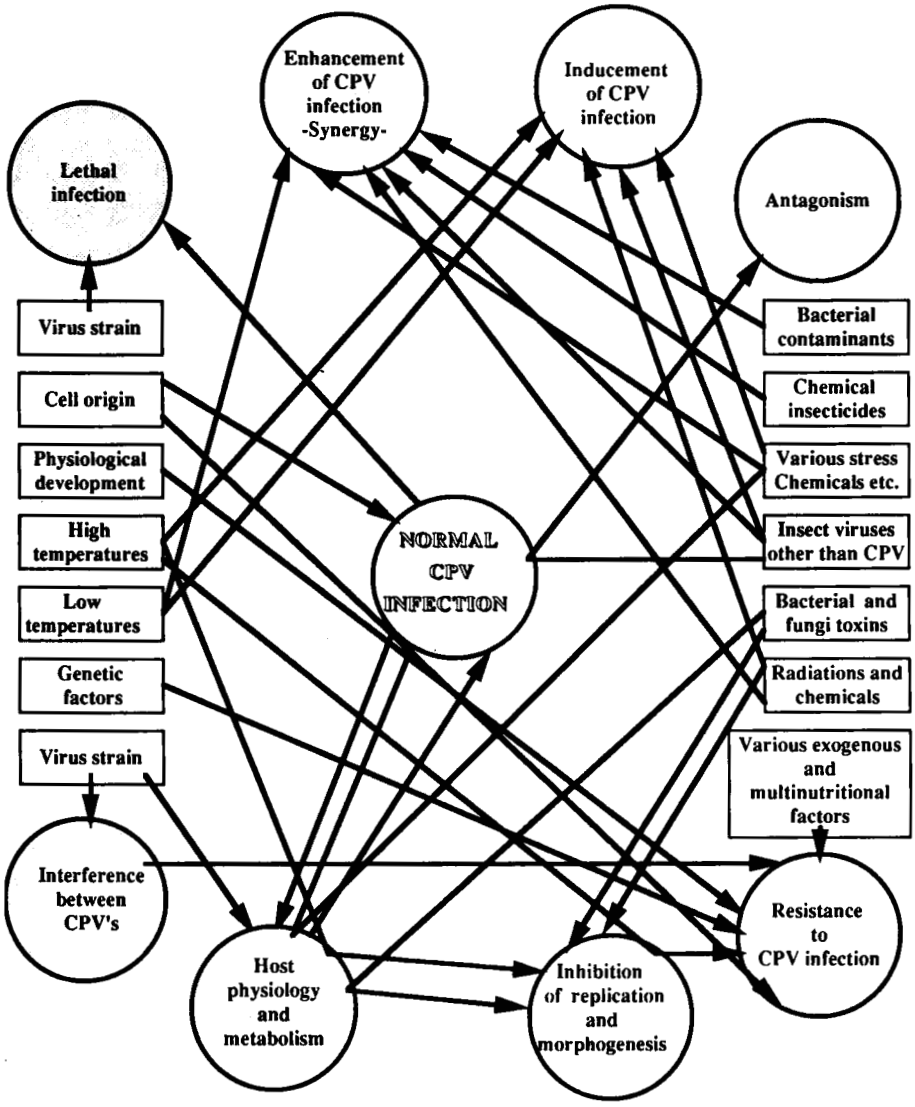

FIG. 11 Complexity of interactions between endogenous and exogenous factor effects on the evolution of CPV infection and pathogenesis.

the virus particle as well as the cell composition are equally important in this viral replication step. In relation to this aspect, we may quote Payne (1981) stating that 'CPV virions contain the enzymatic machinery necessary for the initiation of infection and are not dependent on cellular enzymes for messenger RNA production. Nonetheless, the nucleotide triphosphates and

methyl group donors required for viral RNA synthesis must be provided by the cell. It could be envisaged that the cellular location and availability of these compounds could control the site and the rate of viral RNA synthesis.'

Synthesis of ds RNA of CPV from mRNA could be similar to the process that occurs for Reoviruses (Payne, 1981). High production of ds RNA is noted in CPV-infected midgut (Hayashi and Kawase, 1965) in the absence or presence of Actinomycin D (Kawase and Kawamori, 1968; Hayashi, 1970; Payne, 1972). Two sizes of ds RNA are formed (Hayashi and Donaghue, 1971; Furusawa and Kawase, 1973) and it was demonstrated, by incorporation of labelled uridine, that nuclei are involved in virus ds RNA synthesis (Watanabe, 1967a; Hayashi and Retnakaran, 1970). However, since the last two reports, no confirmation of such nuclear role has been made.

The kinetics of the appearance of viral protein synthesis is partially known. The structural protein of the virus and polyhedrin were detected in midgut at 6 and 9 hours post-infection respectively (Kawase and Miyajima, 1969). When experiments were conducted in cell culture, Arella *et al.* (1984) demonstrated by a 2 h pulse with ^{35}S Methionine and immunoblotting techniques, presence from 24 to more than 96 hours post-infection, of five polypeptides of the polyhedral inclusion bodies and two viral-particle polypeptides. The background of cellular polypeptides was not affected. Therefore, early polypeptide synthesis was difficult to detect and some viral polypeptides were probably not seen because of their low rate of synthesis or because of their migration with other cellular polypeptides. However, the major polyhedral polyhedrin (28K) was easily found from 8 hours post-infection and its rate of synthesis increased with the time course of infection, which suggests the regulation or transcription of viral mRNA for the polyhedrin gene (Arella *et al.*, 1984).

4.2 CPV MORPHOGENESIS

The morphogenesis of CPVs either in insects or in cell cultures has been described in several previous review articles (Kobayashi, 1971; Payne, 1981; Belloncik, 1989; Hukuhara and Bonami, 1992). Therefore, I will, in this section, update and complement these papers with recent information obtained particularly in relation to the interrelations of viruses with cells which characterize CPV infection in insect and in cell culture systems.

Infected insect cell cytoplasm contains polyhedra (Figs 4 and 7) filled with virions and non-occluded viruses. The protein inclusion bodies (polyhedra) are not infectious by themselves. When ingested, they are fragmented and dissolved by appropriate alkaline insect juice (Vago *et al.*, 1959). If these conditions are not met, such as in vertebrate alimentary tract, they are excreted intact and viral replication does proceed further. To infect cell cultures, viral polyhedra must be dissolved in the laboratory, *in vitro*, usually

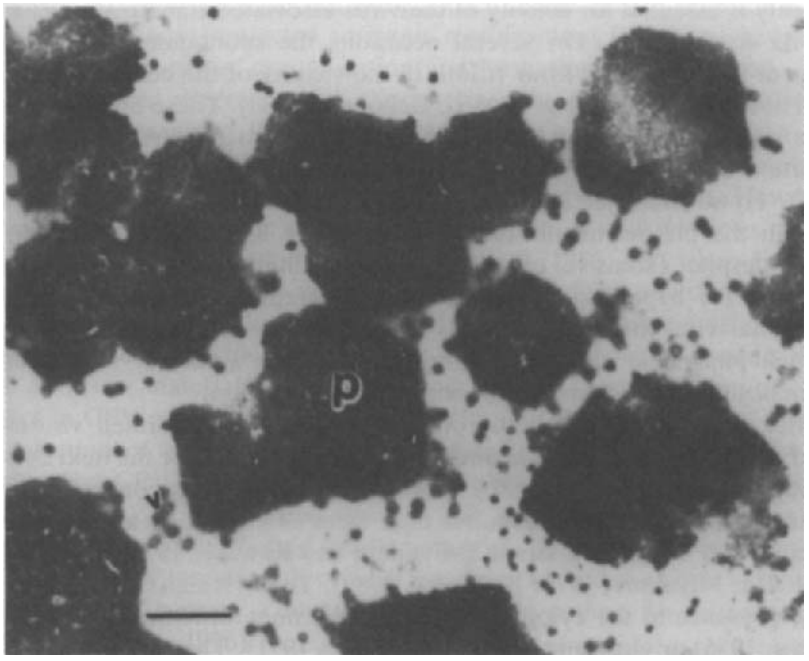

FIG. 12 *In vitro* dissolution of *Es* CPV polyhedra in alkaline buffer. CPV particles released from polyhedra as in insect midgut. Transmission electron microscopy, negative staining. Bar = 700 nm. p = Polyhedra. v = CPV particles.

with sodium carbonate solution pH 10.8 to release the virus particles (Fig. 12) prior to infection. Viruses extracted by this technique are infectious to larvae if injected into the haemocoels (Faust and Cantwell, 1968). Compared with non-occluded viruses they are, in general, equally infectious to cell cultures. However, on some occasions, viral replication is obtained only using virions extracted from polyhedra (Belloncik *et al.*, 1992, 1993, 1994a), which could be due to the high proportion of defective incomplete non-occluded virus particles as observed by Bird (1966).

The early interaction step of virions with the insect cell is not known exactly. According to Kobayashi (1971), the viral core material, after virus adsorption on the cell membrane, is injected through the virus spikes into the cytoplasm of midgut cell without penetration of virion into the cytoplasm. However, this assumption is not acceptable any more since it has been demonstrated that the early interaction event of the CPV virions with cell is pynocitosis or viropexis (Belloncik *et al.*, 1986a; Belloncik, 1989). Further on, virus particles are found in vacuoles (Quiot and Belloncik, 1977) and Lysosome-like structures half an hour post-infection. Moreover, virus particle

integrity is essential for activity of the virus-associated transcriptase (Lewandowski *et al.*, 1969). On several occasions the spontaneous liberation of filament-like structures from virions in the vicinity of the cell membrane has been noted (Belloncik *et al.*, 1986a; Belloncik, 1989). These observations are quite similar to those of Kobayashi (1971), and to mRNA or to ds RNA excretion features from virus particles following UV treatment (Dai *et al.*, 1982). However, injection of such viral material into the cell cytoplasm through the plasma membrane as observed by Kobayashi (1971) was not noted despite extensive examination of ultrathin sections. We advance therefore the hypothesis, which gives some credit to Kobayashi's observations, that viral mRNA excretions in cell cytoplasm from the virus spikes through phagocitic vacuole membranes could be a further step after the internalization of virions, of the virus–cell interaction.

After approximately 3 hours of viral eclipse, newly formed virions are observed in the cell cytoplasm and the number increases for the next 24 hours (Miyajima and Kawase, 1968b). The entire CPV replication cycle is only observed in midgut epithelium but high titres of viral particles are noted in haemolymph of infected larvae (Miyajima and Kawase, 1971; Sikorowski *et al.*, 1971a; Miyajima, 1975; Belloncik, 1989). The first sign of virus infection is the presence in the cytoplasm of small micronet structures as viroplasms (Xeros, 1956) or virogenic stroma (Sohi *et al.*, 1971). They are found initially near the brush border of columnar epithelial cells and thereafter at the base of infected cells. Several viroplasms fuse, later on, to form large matrices of very thin filaments and granules containing RNA and proteins. In these virogenic stroma are the capsids, core and virions and assembly sites of virus particles (Kobayashi, 1971). The mechanism of ds RNA assembly in each virion is unknown. It is suspected that as for reoviruses, no ds RNA of CPV is found free in the cytoplasm (Tyler and Fields, 1985) but within subviral cores where its synthesis occurs (Acs *et al.*, 1971).

The first polyhedra are noted at 15 hours (Quiot and Belloncik, 1977; Payne, 1981) when this protein aggregates (pro-polyhedron) and crystallizes around the virus particles as a polyhedron containing many virions and excluding other cell components. Crystal formation, approximately 6 hours after the initiation of polyhedrin synthesis, is one of the intriguing aspects of the morphogenesis. It is supposed that the crystallization starts when a critical amount of polyhedrin is formed. Belloncik *et al.* (1986c, 1989, 1992, 1993) applied immunocolloidal gold techniques in the studies of CPV morphogenesis. Using polyclonal antibodies directed against alkaline dissolved polyhedra, they succeeded in the specific localization of CPV polyhedrin in cells infected with *Euxoa scandens*, *Heliothis armigera*, *Choristoneura fumiferana* and *Dendrolimus punctatus* CPVs. Interestingly, the gold markers were located, in all the experimental systems tested, either in infected midgut or cell culture, exclusively on the crystal form of polyhedrin. Moreover, the same observations were made when CPV

polyhedrin was expressed in cells using a baculovirus system (Fossiez *et al.*, 1990). These authors suspected antigenic modifications in the polyhedrin at the initiation step of the crystallization and formation of polyhedron.

In the accretion process, which characterizes the polyhedra formation, it was previously assumed that specific recognition between polyhedrin and virus particles exists (Arnott *et al.*, 1968; Payne and Mertens, 1983) implying that polyhedrin crystallizes only when virus particles are present. However, polyhedra formations in the nucleus in the absence of virus particles during infection by the A strain of *Bm* CPV (Hukuhara, 1985) and when Es CPV polyhedrin was expressed in nucleus using the baculovirus system (Fossiez *et al.*, 1990), contradicts this assumption. It is also known that crystallization of polyhedrin is a specific process excluding any foreign components such as cell and virus materials. On only one occasion has incorporation of NPV particles in CPV polyhedra been reported (Inoue, 1983). Otherwise, in dual viral infection of an insect cell, no occlusion in polyhedra of other virus particles than CPV are observed despite the fact that the two viruses share, as in CPV and *Chilo* iridescent virus (CIV), the same morphogenesis site in the cytoplasm of the infected cell (Arella *et al.*, 1983). Moreover, in mixed virus infections of insects with two CPV types, polyhedra of one type were demonstrated to contain a majority of the type coding for its respective polyhedrin (Payne, 1976).

Polyhedra increase in size gradually to a maximum according most probably to virus strain but certainly to host cell and, in particular, to cell environment. In this regard it was noted by several authors that with fractionation in undefined circumstances of dense polyhedra or crystallization of a definite strain in an abnormal inclusion body shape (Fig 13; Hukuhara *et al.*, 1972) polyhedra number varied from one to more than 100 per cell and their sizes from 0.5 to 10 μ. In general, the higher the number of polyhedra per cell, the smaller are their sizes (Longworth and Spilling, 1970; Su *et al.*, 1978). It was also demonstrated that the inclusions formed in the posterior midgut of silkworm larvae (Miyajima and Kawase, 1971) are smaller than those in the anterior part. In addition, the longer the infection time, the more definite the dimension and outline shape of polyhedra obtained.

Different shapes of polyhedra are described: round, cubic, hexagonal, tetragonal, etc. We combined in Figure 14, data published on polyhedrin crystallization patterns and inclusion body descriptions of different CPV strains in insect cells *in vivo* and *in vitro* (Quiot and Belloncik, 1977; Hukuhara and Midorikawa, 1983; Hukuhara, 1985; Belloncik, 1989; Belloncik *et al.*, 1994a). These data indicate that CPVs produce crystal proteins showing a lattice structure reflecting the crystalline molecular organization or on a few occasions inclusion bodies lacking crystalline structure (Hukuhara, 1985). Crystals and inclusions are located in the cytoplasm but on some occasions are found in the nucleus (Fig. 15) following the transport of polyhedrin through a modified nuclear membrane. However, the virus

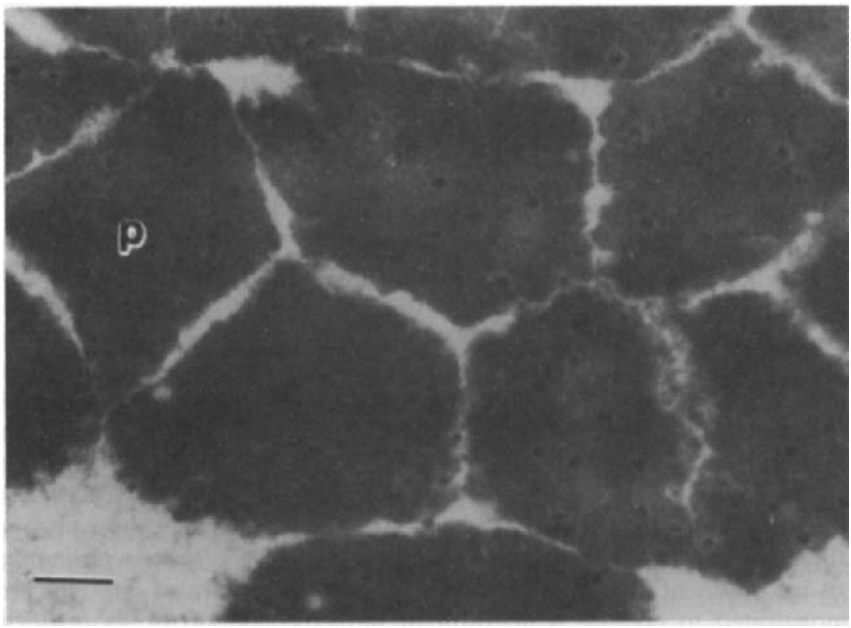

FIG. 13 Abnormal crystallization of *Bm* CPV polyhedrin in embryonic silkworm cell line, fractionation of polyhedra. p = polyhedra. Bar = 300 nm.

particles are occluded only in cytoplasmic polyhedra. Midgut infected with one CPV may show one (Arnott *et al.*, 1968) or several shapes of polyhedra (Federici *et al.*, 1973; Quiot and Belloncik, 1977; Lipa, 1977).

Since polyhedrin is encoded by segment 10 of CPV (McCrae and Mertens, 1983; Hashimoto *et al.*, 1986; Liu *et al.*, 1989), one can ask if the virus is solely responsible for the different shapes and locations of the viral polyhedra observed. Using a mutant CPV strain producing only cubic polyhedra, some experimental data have demonstrated that this shape is maintained after successive passage in larvae (Arnott *et al.*, 1968). Furthermore, the differential polyhedrin amino acid composition coded by each strain of *Bm* CPV was shown to influence not only the location but also the crystallization pattern of polyhedrin (Miyajima and Kawase, 1971). It was also demonstrated that mutations in amino acid sequences of *Bm* CPV strain H polyhedrin which produce polyhedra in cytoplasm lead to intranuclear transport and crystallization of polyhedrin typical to *Bm* CPV strain A (Mori *et al.*, 1989). Therefore, the preponderant role of the virus in the formation of polyhedra was quite evident. However, the intervention of the cell in this latter process was suspected more than twenty years ago. Miyajima and Kawase (1971), as well as Hukuhara (1971) proposed a hypothesis in relation to the variations in polyhedra sizes and shapes. These authors found interactions between several

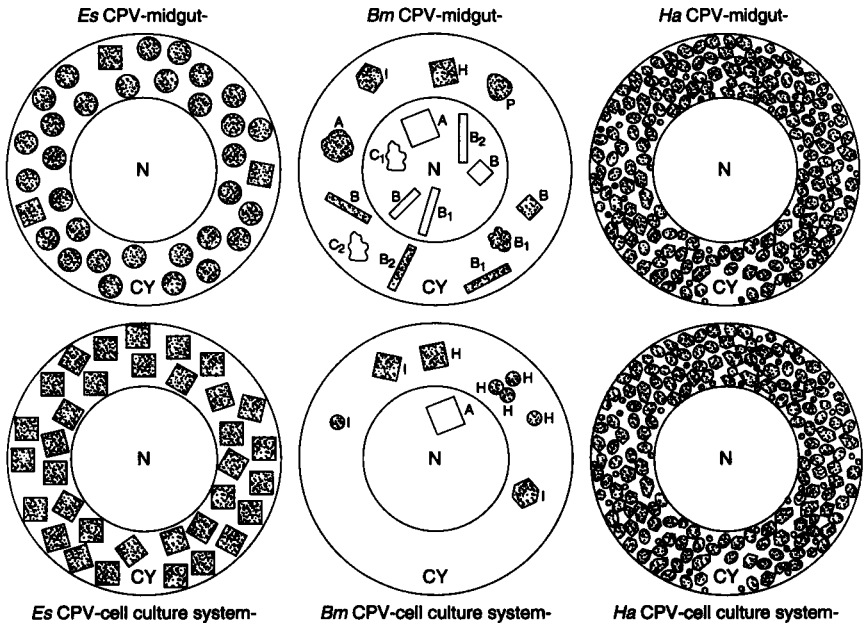

FIG. 14 Three representative patterns of CPV polyhedrin crystallization in insect midgut and cell culture systems.

Bm CPV = *Bombyx* CPV, different strains: H, A, B, B_1, B_2, C_1, C_2, P, I.
Es CPV = *Euxoa scandens* CPV.
Ha CPV = *Heliothis armigera.*

factors, such as metabolism of the different midgut cells, nutrition, stress, larval age, etc., and CPV strain, which result in specific final crystallization pattern of polyhedrin. Hukuhara (1971) clearly pointed out the question when he compared the crystallization patterns of polyhedrin to those of ferritin. As for ferritin, the crystal shape is influenced by the exogenous conditions under which crystallization occurs. Changes of the cellular environmental conditions under virus control will influence the polyhedrin crystallization.

Later on, Quiot and Belloncik (1977) noted in *E. scandens* CPV-infected midgut cells the presence of spherical and cubical polyhedra accounting for 10 and 90% respectively of the polyhedra. When passed in *L. dispar* and several other cell cultures (Quiot and Belloncik, 1977; Belloncik *et al.*, 1989), only cubical polyhedra were formed. When larvae were fed with cubic polyhedra obtained from cell culture the two shapes reappeared and so on for several cross-infection experimentations *in vivo–in vitro* (Belloncik and Bellemare, 1980). Furthermore, similar observations were made when numerous other cell culture systems were used (Table 1; Figs 16, 17, 18) and

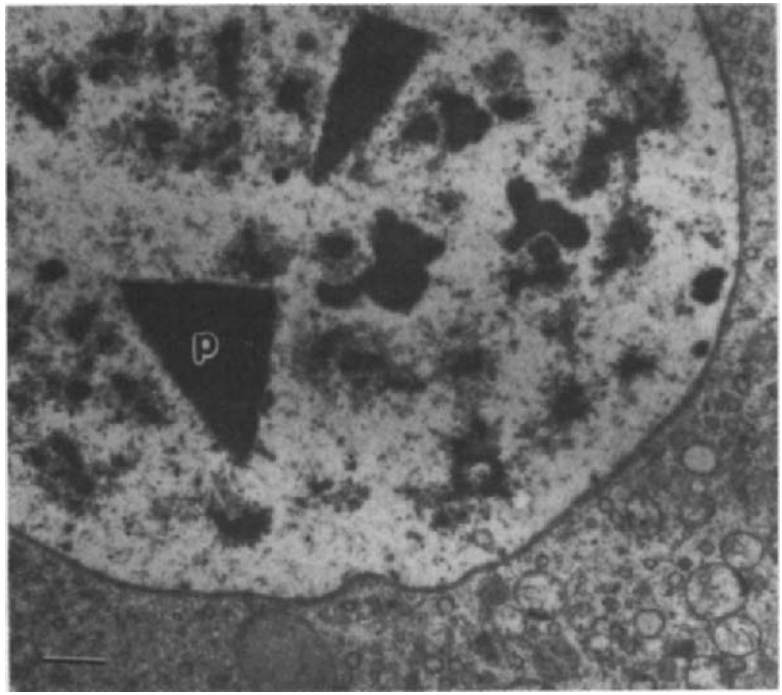

FIG. 15 Intranuclear location of inclusion bodies of strain A of *Bm* CPV in silkworm cell line. Note the absence of viral particle. Electron microscopy. Bar = 700 nm.

we found that the initial form of the inclusion bodies is spherical, but they become cubic at a late infection stage (Belloncik, 1989). For one strain of CPV such as *Bm* CPV, the cubic mature form is achieved in only some particular cell lines and, moreover, in only some cells of this cell line and not in others (unpublished). For other CPV strains the cubic form is rarely noted (Fig. 20). The conclusion of these observations is that the final form of the polyhedron is the cubic one. In the insect midgut and in different cell cultures, undetermined cell conditions, interacting with the virus strain, are required for a full maturation of inclusion bodies. This conclusion could be in accord with the hypothesis of Hukuhara (1985) who stated that the shapes 'of mature inclusion bodies may be the equilibrium forms. . . . Before the equilibrium has been reached, the inclusion bodies may exhibit the growth forms and their shapes may change during the crystal growth'. Moreover, we suspect that the rate of transport of polyhedrin to the nucleus during some CPV infections (Figs 15 and 18) and consequently the differential locations of polyhedra in the cytoplasm and nucleus could reach an equilibrium status between these two cell compartments.

Expression of the *B. mori* polyhedrin gene in *E. coli* resulted in the formation of abnormal insoluble inclusion bodies (Lavallée *et al.*, 1993). However, expression of the same (Mori *et al.*, 1993) as well as *Es* CPV polyhedrin genes (Fossiez *et al.*, 1990) in insect cell culture using baculovirus expression system resulted in normal nuclear cubic polyhedra. These data could indicate that some cell factors or conditions present in eukaryotic cells are necessary in the process of polyhedra maturation. Whether such factors are only present in insect cells or if polyhedrin is able to crystallize in plant or vertebrate cells merits investigation. Nevertheless, the influence of the nature of the cell on polyhedrin crystallization is very important to consider for a better understanding the CPV specificity (see later).

4.3 INFLUENCE OF SOME EXOGENOUS FACTORS ON CPV REPLICATION AND MORPHOGENESIS

CPV replication and morphogenesis are greatly enhanced, or affected, by the nature of host cells and their physiological status. In addition, numerous exogenous factors will influence these events during viral infection. Interestingly, several chemical or chemical-like and physical factors which have been demonstrated to inhibit virus infectivity, may also demonstrate, in certain conditions, an enhancement effect on CPV replication or inducement. However, these contradictions are explained when we know that these factors act on the virus particle integrity as well as on the insect cell physiology and metabolism. This latter effect could be responsible for the enhancement of CPV infection noted during several experiments.

4.3.1 *Chemical factors*

Gut juice and carbonate buffers responsible for dissolution of polyhedra and liberation of occluded virions are crucial events for the initiation of virus replication *in vivo* (Vago *et al.*, 1959; Aruga and Watanabe, 1964) and *in vitro* (Quiot and Belloncik, 1977; Belloncik *et al.*, 1994a) respectively. However, gut juice was demonstrated to have an antiviral effect (Aratake, 1974). In addition, formalin (Bullock *et al.*, 1969) and calcium hydroxide (Aruga, 1971) reduce the virus infectivity when CPV particles are treated with these substances. However, when *B. mori* larvae are exposed to formalin (0.01–1%) and other products such as H_2O_2 (Aruga, 1963), sodium azide, EDTA, 0.1 M (Hukuhara, 1961), disodium salts (Aruga, 1971), nitrogen mustard (Aruga and Hukuhara, 1960; Yokokawa and Yamagushi, 1960), boric acid (review of Katagiri, 1981), silica, and ether for 5–10 minutes, the incidence of CPV infection is greater. It was also demonstrated that chemical insecticides (Watanabe, 1971b; Aratake *et al.*, 1973) and different dietary ingredients (Kunimi and Aruga, 1974) increase the susceptibility of silkworms to CPV. More recently significantly higher mortalities were recorded when

TABLE 1 Replication of cytoplasmic polyhedrosis viruses in cell culture system: major achievements 1962–1994

CPV strain	Cell culture identification	Origin	References*
Ae. sollicitans	*Ae. albopictus*		Barry and Fowler (unpublished, quoted in Granados, 1976)
B. mori	*B. mori.* -primary culture-	Midgut epithelium	Kobayashi, M., 1971
	L. dispar. SCLd-135	Pupal ovaries	Hukuhara and Midorikawa, 1983
	B. mori. Bm N4	Pupal ovaries	Kobayashi, J., 1993
	B. mori. BoMo-15AIIC	Embryos	Belloncik *et al.*, unpublished.
Unknown: Viral inoculum: Bm NPV	*A. eucalypti*-primary culture-	Pupal ovaries	Grace, 1962
Unknown: Spontaneous CPV infection	*A. eucalypti* 10 month subcultures	Pupal ovaries	Grace, 1962
C. eriosoma	*S. frugiperda* IPLB-SF-21	Pupal ovaries	Longworth, 1980
	T. ni TN-368	Adult ovaries	Longworth, 1980
C. fumiferana	*S. frugiperda* Sf9	Pupal ovaries	Belloncik *et al.*, 1992, 1993
D. punctatus	*B. mori.* BoMo-15AIIC	Embryos	Belloncik *et al.*, 1992
D. punctatus	*B. mori.* Bm N4	Pupal ovaries	Belloncik *et al.*, unpublished
D. punctatus	*S. frugiperda.* Sf-9	Pupal ovaries	Belloncik *et al.*, 1993
E. biplaga	*E. scandens* IAF-Es-1	Pupal ovaries	Belloncik *et al.*, 1984b

E. scandens	*L. dispar* SCLd-135	Pupal ovaries	Quiot and Belloncik, 1977
	C. fumiferana IPRI Cf 124	Larval haemocytes	Arella *et al.*, 1984
	*T. ni.*TN 368	Adult ovaries	Arella *et al.*, 1984
	A. eucalypti SBM **	Pupal ovaries	Arella *et al.*, 1984
	M. disstria. Md 66	Larval haemocytes	Arella *et al.*, 1984
	P. xuthus Px-58 Si	Pupal ovaries	Arella *et al.*, 1984
	E. scandens IAF-Es-1	Pupal ovaries	Belloncik *et al.*, 1984b
	O. nubilalis -primary culture-	Larval haemocytes	Rocheleau, 1987
	B. mori. -primary culture-	Larval haemocytes	Belloncik, 1989
	G. mellonella -primary culture-	Larval haemocytes	Belloncik, 1989
	H. armigera Ha 831	Larval haemocytes	Belloncik *et al.*, 1989
	E. scandens	Larval haemocytes	Belloncik, unpublished
	B. mori. SES-BoMo-15A	Embryos	Inoue and Belloncik, 1990
	S. frugiperda Sf 9	Pupal ovaries	Belloncik and Akoury, 1993
H. armigera	*H. armigera* Ha 831	Larval haemocytes	Su *et al.*, 1978; Belloncik *et al.*, 1994a
I. io (Type 2)	*S. frugiperda*	Pupal ovaries	Payne (unpublished, quoted Payne and Mertens, 1983)
L. dispar	*L. dispar* -primary culture-	Pupal ovaries	Vago and Bergoin, 1963
M. disstria	*B. mori* -primary culture-	Ovaries and trachea	Sohi *et al.*, 1971
	A. eucalypti	Pupal ovaries	Karawabata and Hayashi, 1971
T. ni	*T. ni.* TN-368	Adult ovaries	Granados *et al.*, 1974
	S. frugiperda IPLB 21 A	Pupal ovaries	Granados, 1976

*See complete title in the reference section.
**Original designation *B. mori* SPC Bm-36.

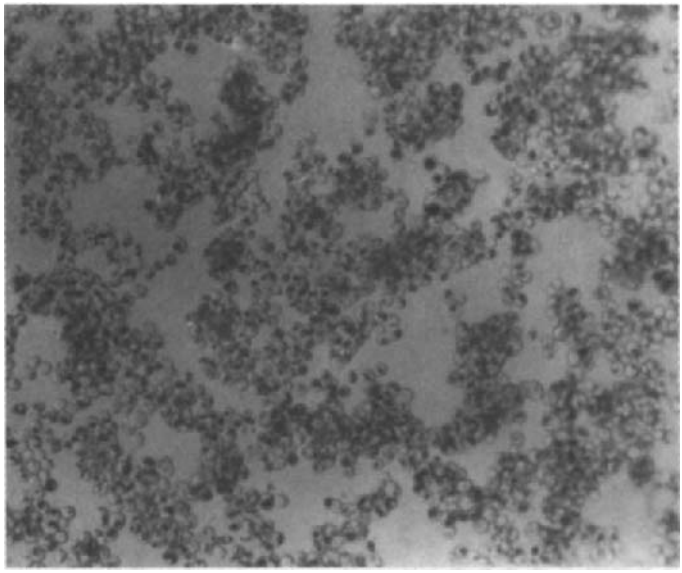

FIG. 16 General aspect of *E. scandens* cell line infected with *Es* CPV. Light microscopy. The black spots are polyhedra.

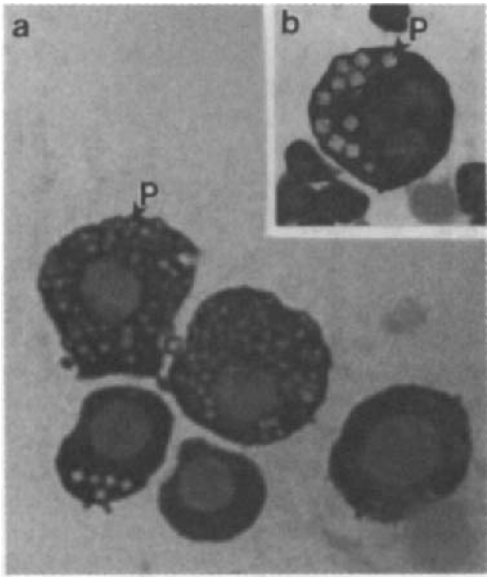

FIG. 17 High number (a) and cubic shape (b) of *Es* CPV polyhedra (P) in *Sf*-9 cell line. Light microscopy. P: polyhedra.

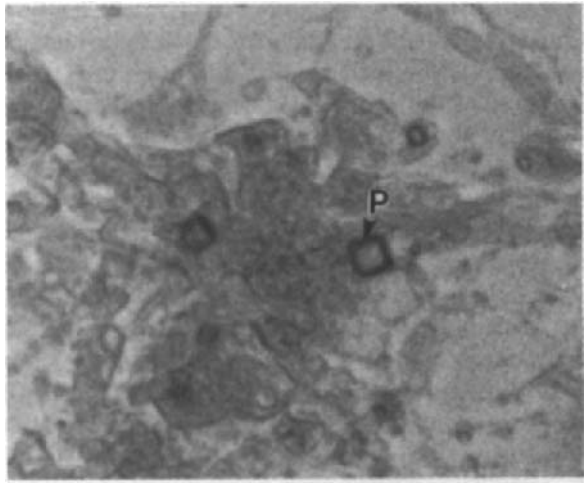

FIG. 18 Infection of silkworm cell line with A strain of *Bm* CPV. Light microscopy. Observation after 28 months post-infection without medium change and supply. Note the low infection rate. One or two large polyhedra characterize the replication *in vitro* of this CPV. P: polyhedra.

E. scandens larvae were treated with *Es* CPV prior to permethrin (Rud and Belloncik, 1984). However, these results may be explained by an increase in sensitivity of CPV-infected larvae to this chemical insecticide rather than enhancement of *Es* CPV replication.

Toxins from entomopathogenic bacteria and fungi have differential effects on viral replication. Crystallization of polyhedrin and consequently the number of polyhedra are affected by Cordycepin produced by the fungi *Cordyceps militaris* (Belloncik and Arora, 1979) along with an inhibition at higher toxin dose of CPV RNA synthesis (Truong, 1990). Synthesis *in vitro* of viral RNA is also affected by δ endotoxin of *B. thurigiensis* (Truong, 1990). On the other hand destruxin produced by *Metarizium anisopliae* has no effect on viral RNA synthesis but has detrimental effects on the polyhedra formation and maturation (Belloncik and Kato, in preparation).

4.3.2 *Physical factors*

4.3.2.1 *Radiation.* Treatment of CPV with ultraviolet radiation results in inactivation of virus infectivity (Aruga and Hashimoto, 1965; Aruga and Watanabe, 1970) and may disrupt virus particle structure (Dai *et al.*, 1982). However, UV as well as X-rays were demonstrated to induce cytoplasmic polyhedrosis in *B. mori* (Aruga and Yoshitake, 1961). More recently, exposure of CPV-infected cells *in vitro* to gamma radiations resulted in an increase of CPV polyhedra number, as a consequence of excessive CPV

polyhedrin synthesis, together with abnormal crystallization of this viral protein (Belloncik and Arella, 1981). Interestingly, higher numbers of polyhedra were also noted when NPV-infected cells received a similar treatment (Koval and Hink, 1977). Imbalances of protein synthesis in γ irradiated cells could be responsible for these observations.

4.3.2.2 *Temperatures.* Heat treatment has been investigated as a prophylactic approach to CPV control (Tanada, 1967). Inactivation of CPV infectivity could be achieved by virus particle treatments at 80°C or at 60°C for 10–15 minutes and 140–160 minutes respectively (Aruga *et al.*, 1962). Incubation of insects and cell cultures at temperatures between 23 and 28°C is considered optimal for complete normal CPV replication. At higher temperatures, viral RNA synthesis in larvae is inhibited (Kobayashi and Kawase, 1980) which could decrease and suppress the disease incidence in larval colonies reared at 35 or 37°C (Tanada, 1967; Aratake *et al.*, 1968; Miyajima and Kawase, 1968a; Tanada and Chang, 1968).

Data obtained *in vitro* corroborated those obtained *in vivo*. Virus adsorption was enhanced when infected cells were maintained for up to three hours at 37°C (Rocheleau, 1987). However the virus replication was thereafter inhibited if the cells were maintained exclusively at this temperature. No protein and RNA synthesis was noted at 37°C and the rate of inhibition was a function of duration of this treatment. The shorter the CPV-infected cells were incubated at 28°C before being switched to 37°C, the higher was the inhibition of infection. It is not known exactly for how long the virus infectivity is preserved in cells maintained at 37°C. However, if infected cells were incubated at 37°C and thereafter at 28°C (Truong, 1990; Belloncik and Truong, 1991), viral infection was demonstrated to resume but with fewer polyhedra being formed. These authors demonstrated also that incubation at 37°C for 14 days of cells cultivated *in vitro* prior to CPV infection at 28°C reduces by 50% the number of CPV polyhedra. Therefore, high temperatures act negatively not only on virus replication but also on the cell host, which in turn alters CPV morphogenesis. In larvae, however, it was shown that heat treatments of larvae induce CPV infection (Aruga, 1971) and that special tissues in the anterior part of the larva subjected to high temperatures are responsible for the viral induction phenomenon (Hukuhara and Aruga, 1959). Moreover high temperature has an effect on moulting behaviour of insects, which in turn influences the expression of CPV infection.

Low temperatures such as 4°C were frequently associated with activation or inducement of CPV infection in *B. mori* larvae (Tanada, 1971). However, no data were published on the quantitative relation between the decrease of rearing temperature and viral replication. Recent studies conducted *in vitro* showed that the number of *Es* CPV polyhedra decreased with the incubation temperature of infected cells *in vitro*. However, at 4°C, virus

infectivity was preserved and, moreover, incomplete virus replication, such as ds RNA without virus protein synthesis, occurs (Truong, 1990; Belloncik and Truong, 1991). This replication pattern, which it is important to consider in the biological control of insects, is currently under investigation in our laboratory.

Studies on the influences of non-optimal temperatures on polyhedra integrity complement those on the effects of these temperatures on viral replication. It was demonstrated that temperature has differential effects on crystal formation according to CPV strain. High temperatures such as 37°C and 34°C have no effect on the crystallization pattern of the polyhedrin of *Es* CPV (Truong, 1990; Belloncik and Truong, 1991) as well as *Bm* CPVs, strains H, B, and B_1 (Hukuhara, 1985) respectively. However, high temperature affects the shape of strain A polyhedra (Yamaguchi *et al.*, 1969) and their shape returns to normal if the temperature is switched back to the optimal one. Furthermore, Hukuhara and Yamagushi (1973) observed decomposition, at incubation temperatures of 30° and 35°C, of cytoplasmic and nuclear polyhedra of this latter CPV strain. On the other hand, maturation of inclusion bodies of strains C_1 and C_2 of *Bm* CPV was enhanced if the incubation temperature was switched from 25 to 20°C (Yamaguchi and Hukuhara, 1973; Hukuhara, 1985). Furthermore, recent studies demonstrated that polyhedrin of *Bm* CPV expressed in *E. coli* accumulates in an insoluble form at 42°C (Lavallée *et al.*, 1993) but that clear crystalline arrangement appeared when bacterial cells or inclusion bodies were stored at below 4°C (−20°C) for one and three months (Lavallée, 1991). This author explained the phenomenon by an organization of polyhedrin molecules in a crystal structure in the presence of ice crystals formed at −20°C.

To conclude this section on virus morphogenesis, the importance is emphasized of the phenomenon of interaction between the virus strain and the cellular and external conditions during CPV morphogenesis and, in particular, on polyhedrin crystallization. More studies are warranted to elucidate the nature of these interactions and the role of the different CPV genes.

4.4 VIRUS SPECIFICITY: SUSCEPTIBILITY AND RESISTANCE OF INSECTS TO CPV INFECTION

4.4.1 *Role of the insect*

CPVs demonstrate a wide host range among Lepidoptera (Tanaka, 1971; Belloncik *et al.*, 1980; Katagiri, 1981). However, no cross-viral infection has been observed between other insect orders. Moreover, different biosafety studies conducted in several animals (Granados, 1978; Katagiri, 1981; Belloncik *et al.*, 1987; Chen, 1990) demonstrated no susceptibility of

vertebrates to CPV infection. Other recent studies conducted in our laboratory have demonstrated also the absence of the replication of *Es* CPV in different vertebrates as well as the absence of CPV genome integration and persistence in mosquito and vertebrate cells *in vivo* and *in vitro* (Belloncik *et al.*, 1986b; 1987). The main explanation of this lack of susceptibility of vertebrates is the block of CPV replication at 37°C owing to the non-activity of the viral transcriptase at this temperature and the absence of appropriate conditions for the dissolution of polyhedra in the vertebrate digestive system.

Complete replication of CPV leading to the formation of virions and mature polyhedra is detected only in midgut epithelial cells (Fig. 7). The target cells are the same if polyhedra are ingested by larvae or if virions, non-occluded or extracted from alkali dissolved polyhedra, are injected into the larva haemocoel (Faust and Cantwell, 1968; Miyajima and Kawase, 1971; Belloncik, 1989). A few days post-infection, high titres of virions are detected in the haemolymph (Miyajima, 1975; Sikorowski *et al.*, 1971a; Belloncik, 1989) and despite that, haemocytes and other cells, including those of the fat body, ovaries, hypoderm, various glands, etc., are polyhedra free. Columnar cells are the main infected cell type. But, on rare occasions, polyhedra have been observed in goblet cells of *Orgyia pseudotsugata* (Martignioni *et al.*, 1969) and *B. mori* (Iwashita, 1971) midgut. Regenerative cells in the basal region of the epithelium are infected only after their differentiation and maturation. An absence of a substance or a receptor site, in undifferentiated regenerative cells, necessary for CPV replication could explain these observations (Iwashita, 1971). Moreover, when CPV was inoculated into *B. mori* embryos through the egg micropyle (Kitazawa and Tatami, 1959), or by immersion in virus solution, of silkworm embryo surrounded by serosa (Takami *et al.*, 1967), CPV polyhedra were found in embryonic midgut at the end of embryonic development. Morphological and functional differentiation of midgut epithelium was concluded as necessary for CPV multiplication (Takami *et al.*, 1967; Iwashita, 1971).

In the light of this infection feature, we can conclude that CPV infection is specific to the epithelial midgut cells. However, the notion of CPV virus specificity must be reassessed following data obtained recently, First, haemocytes from infected larvae, which do not support polyhedra formation even though the haemolymph contains virions, develop the entire normal viral infection if transferred to a Falcon plastic flask containing Grace's insect culture medium (Belloncik, 1989). Second, in contrast to insect cells *in vivo*, primary and established cell cultures of insects do not demonstrate any degree of viral specificity. Several types of insect cells, such as those of the ovary, embryo, haemolymph in culture, have been demonstrated to be equally susceptible to CPVs (Table 1). We can draw these conclusions from the observations. 1. Some antiviral substances present in larvae inhibit penetration or the entire replication of the virus in tissues other than those of midgut.

2. Some factors and conditions in cells, except those of midgut, are not favourable for the synthesis of viral proteins or for the crystallization of polyhedrin, if synthesized. Therefore, experiments are presently being conducted in our laboratory to know exactly the specificity of virus infection in larvae. Particularly, we are investigating the possibility that cells of larva except those of midgut could, in contrast to the current belief, support a partial CPV replication cycle as demonstrated *in vitro* where synthesis of viral ds RNA take place without the synthesis of virus particles and the expression of polyhedrin and therefore without the formation of polyhedra (Truong, 1990; Belloncik and Truong, 1991; Kobayashi and Belloncik, in preparation). The application of molecular biology as well as genetic engineering technology will contribute to the elucidation of some aspects of CPV specificity.

Differences in the virus cell receptor at epithelium of midgut and peritrophic membranes may underlie the resistance mechanisms to CPV infection (Watanabe, 1971b). Moulting has also been demonstrated to have an inhibitory effect on the development of CPV (Watanabe, 1971b).

Several studies, conducted mainly in *B. mori*, have demonstrated a differential susceptibility or resistance of a particular insect to CPV disease. For example Daizo and Okusa strains of *B. mori* are resistant and the most susceptible to CPV infection respectively. Cross-experiments between silkworm strains indicate that the resistance is under a multifactorial genetic (Watanabe, 1971b) or a dominant major gene control system (Watanabe, 1965).

Insect populations, in nature, may develop resistance to insect viruses. For example, insect resistance to NPV after sublethal infection of *C. fumiferana* (Bergold, 1951). The same phenomena may occur when insect populations are exposed to CPV. Watanabe (1967b) demonstrated that selection pressure following CPV treatment contributes to the retention of more resistant *B. mori*. Moreover, resistance to CPV was also demonstrated following the breeding of the survivors of a GV treatment (Sidor, 1959).

The older the larvae are, the less susceptible to virus infection they are (Aruga and Watanabe, 1964; Bird, 1969; Aruga, 1971; Bellemare and Belloncik, 1981; Sikorowski and Lawrence, 1994). Nutrition of larvae influences the infectivity of cytoplasmic polyhedrosis viruses. The composition, as well as quality, of diet ingredients can influence the evolution in larvae of CPV infection (Watanabe, 1971b).

The same phenomena of CPV susceptibility and resistance are noted in insect tissue culture systems, even though virus specificity is not restricted to midgut cell cultures (Table 1). Vertebrate and mosquito cells maintained at 28°C are demonstrated to be resistant to replication of Lepidopteran CPV even though virus particles are internalized in these cells (Belloncik *et al.*, 1986b). Numerous experiments conducted *in vitro* demonstrate (Rocheleau, 1987; Belloncik *et al.*, 1989, 1993) that different lepidopteran cell lines are

resistant to CPV infection and moreover in one cell line originating from the same tissue or organ, some cells are resistant to CPV and others are not. Cloning of insect cells *in vitro* has resulted in the selection of more susceptible cells to CPV infection (Rocheleau, 1987). However, after several subcultures parental cell characteristics reappeared.

4.4.2 *Influence of the virus strain*

4.4.2.1 *CPV virulence: lethal and chronic infections.* The reaction of an insect *in vivo* to CPV in terms of infection and lethality is variable according to several parameters such as the virus strain, insect species and larval development stages, as well as microbial contaminations and other extrinsic environmental conditions.

Data, reviewed by Payne (1981), have been published in relation to median lethal and EC_{50} infective doses for numerous CPVs. These data, and more recent studies in our laboratory, demonstrate that the lethality associated with CPV is highly variable according to the CPV strain. CPVs are highly infectious to insect larvae and some CPVs are more infectious than other occluded viruses such as NPVs (Katagiri, 1981). However, in general, the CPV infection is more a chronic than a lethal disease. Larvae even though very infected may not die and can reach pupal and adult stages. Severe alterations other than lethality affecting the insect lead to a final global important decrease in the insect population density (Bellemare and Belloncik, 1981; Katagiri, 1981; Payne, 1981).

In general, CPV will be more infectious if the virus particles are injected into the haemocel rather than ingested by larvae (Faust and Cantwell, 1968). Different strains isolated from several ecological and geographical areas have been demonstrated to have variable virulence for one insect species (review of Katagiri, 1981). The virulence of a particular CPV strain for an insect species that is not its natural host increases when serial passages of the virus are made in this host (Tanaka, 1971; Katagiri, 1981; Belloncik *et al.*, 1984a). However, the virus may lose virulence for its original insect species when passaged in an alternate one (Tanaka, 1971).

Viral strain, age of larvae and route of inoculation combined with other exogenous conditions influence infection and mortality rates of insects. It is important to point out that lethality of one CPV strain evaluated in the laboratory could be very different from what really happens in nature where larvae are subjected simultaneously to several and various detrimental conditions. It has been demonstrated that some viruses such as *Es* CPV, even though highly infectious, are not lethal to *E. messoria* older than the second larval instar (Bellemare and Belloncik, 1981) when reared in the laboratory on artificial diet. However, *Es* CPV infected third instar larvae of *E. messoria* succumb in pilot field experiments (Quévillon, 1984). This is different from

other CPVs, such as those of *Bm*, *Dendrolimus* and *Choristoneura fumiferana* which are highly lethal in any condition.

4.4.2.2 *CPV strains relationships.* Studies on the interactions between different CPVs and on the possibility that CPV-infected larvae develop resistance to further cypovirus contamination have been conducted. Apart from the enhancement of alfalfa CPV with *Bm* CPV observed by Tanada and Chang (1964), antagonisms between CPV infections were more frequently noted. Therefore, this research aspect has been largely investigated to develop prophylactic methods for the prevention of CPV infection in the silkworm-rearing industry (Aruga *et al.*, 1963). More details related to these studies are given in Tanada's review (1971). It was noted more than 30 years ago that CPV infection induced by a stressor interferes with a further CPV inoculation (Aruga *et al.*, 1961; Aruga and Yoshitake, 1962). More recently, we observed on some occasions the ineffectiveness of *C. fumiferana* CPV for larvae of this insect reared in our laboratory and chronically infected with *cf* CPV. Differentiated regenerated midgut cells, which are refractory to CPV, are also found on some occasions following a primary CPV infection (Yamaguchi, 1979). From the results of laboratory experiments (Aruga *et al.*, 1961; Tanada, 1971) it was concluded that interference between CPV infections in larvae takes place under the different conditions discussed in section 4.4.3. Interference will develop not only between CPV strains belonging to the same type, such as between several strains of *Bm* CPV (type 1) or between *Bm* CPV and *Dendrolimus spectabilis* CPV (type 1), but also between *Bm* and *Es* CPV classified as types 1 and 5 respectively (Miyajima and Belloncik, 1993). Interestingly, active infective virus particles are not necessary for the interference process to occur since UV (Aruga and Hashimoto, 1965; Aruga and Watanabe, 1970) or heat-inactivated (Aruga *et al.*, 1963) viruses also prevent further infection with active challenge virions.

Experimental studies conducted in larvae demonstrate that the interactions between CPV strains in insect larvae are very complex phenomena. Different factors such as the insect genetic make-up, nature of the viral strain, larval instar, infection route, environmental conditions and sequence of applications of the viruses all influence the results of co-infection of two CPV strains (Tanada, 1971; Miyajima and Belloncik, 1993).

At a cellular level, despite several reports on insect infection with one or several CPVs (Aruga *et al.*, 1961; Tanaka and Aruga, 1967; Yamaguchi, 1974; Payne, 1976; Belloncik *et al.*, 1984a, 1992, 1993, 1994a), it was admitted, based on observations of the presence of polyhedra, that each CPV strain replicates, during mixed infections, in separate cells of the midgut (Aruga *et al.*, 1961; Tanaka and Aruga, 1967; Yamaguchi, 1974). Therefore, the presence of a resistance mechanism of a CPV-infected cell to a further CPV infection was suspected. However, recent interaction studies between CPV

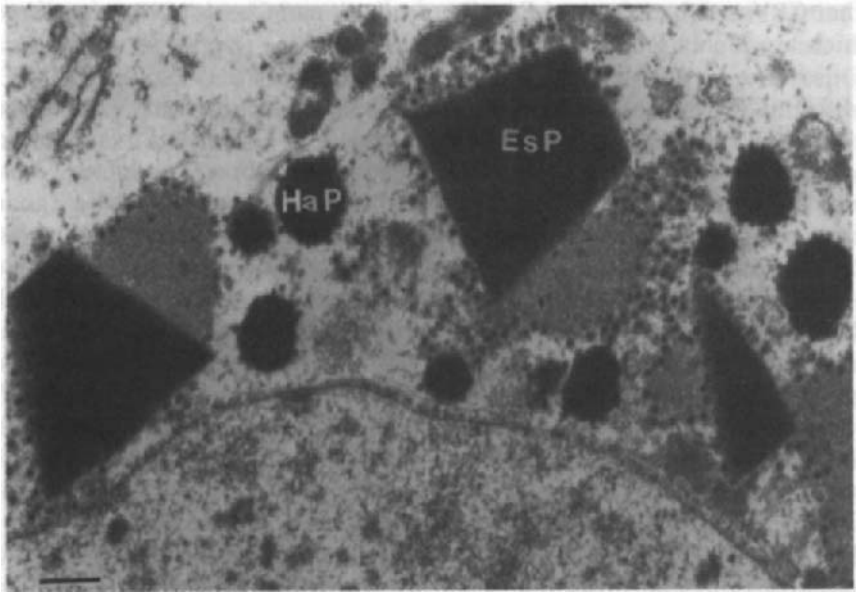

FIG. 19 CPV replication in *H. armigera* cell culture. Note the cubical and spherical shapes of *Es* and *Ha* CPV polyhedra respectively. Electron microscopy. EsP: *Es* CPV polyhedra. HaP: *Ha* CPV polyhedra. Bar = 1 μm.

strains, conducted *in vitro*, demonstrate mixed infection of a cell line with three different CPVs (*Bm*, *Es* and *Ha* CPVs). Moreover, a single cell was able to support (Fig. 19), in certain conditions, simultaneous replication of two different CPV types, *Es* and *Ha* (Belloncik, 1989). Successful evolution *in vitro* of the dual CPV infection was obtained when *Es* CPV infection was already developed at the time of the introduction of *Ha* CPV (Belloncik, 1989).

It is not clear whether interferon-like substances are produced in insects. Competition between CPVs at cell receptor or at metabolic pathway levels are more likely to be responsible for the differential interactions observed. It will be important to investigate further the physiological changes in cells induced by CPV infection and its effects on the replication of other CPVs or pathogens.

4.4.2.3 *Expression of susceptibility and resistance of an insect to CPV infection as a result of the integration of the insect and virus influences.* In summary of this section, I may point out that one condition which influences the susceptibility and resistance of an insect to CPV infection acts at the penetration level of viruses in the cell. However, other numerous genetic and

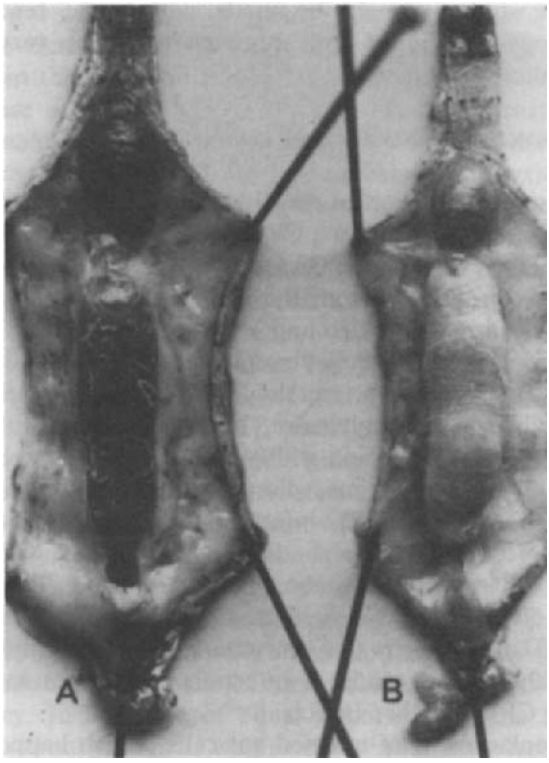

FIG. 20 Appearance of *E. scandens* gut of larva infected with *Es* CPV (B). Control: (A).

external conditions, such as temperatures, prevent CPV replication and consequently act indirectly on the capacity of an insect to resist or to be susceptible to the viral infection. Later on, the physiology of the insect, as well as several exogenous factors will control, by their interactions, the progression or regression of the CPV infection in the insect.

However, most conclusions on insect susceptibility and resistance to CPV infection as reviewed up to now have been based on the presence or absence of viral polyhedra respectively. The interpretation of these observations must be reassessed according to recent knowledge acquired on CPV replication. Incomplete replication of CPVs occurs in different cell systems and conditions. For example, during co-infection of an insect with two types of CPV, a single polyhedrin could be expressed (Payne, 1976) and the absence of protein synthesis characterizes replication of CPV in some cell systems (Kobayashi and Belloncik, in preparation) and at low temperature (Truong, 1990; Belloncik and Truong, 1991). Therefore conclusions related to insect

cross-protection studies should be made with some reservations. New molecular biology techniques and approaches will be very useful in the elucidation of these problems.

4.5 TRANSMISSION AND PERSISTENCE OF CPV INFECTION

In the early stage of infection of most insects, the posterior portion of the midgut is infected, and later on the CPV infection moves to the anterior part (Aruga, 1957). However, on one occasion, it was reported that transmission of CPV infection proceeded from the anterior to the posterior midgut of *Cactoblastis cactorum* (Tamashiro and Huang, 1963). At an advanced stage of infection, cytoplasm of epithelial midgut cells is heavily filled with various viral synthesis products such as: ds RNA, non-occluded virions, non-crystallized polyhedrin and polyhedra. Infected columnar cells will remain intact or their plasma membrane will lyse under the pressure of polyhedra (Kobayashi, 1971). At this time, diarrhoea is common and is the most representative symptom of CPV infection. Intact and lysed infected cells desquame and infectious viral polyhedra (Saito and Yamaguchi, 1960) as well as non-occluded viruses (Watanabe, 1968) are released in insect faeces (Boucias and Nordin, 1978). A decrease in the CPV-infected gut pH (Watanabe, 1971a) will help the evacuation of intact polyhedra in faeces (Vago *et al.*, 1959). This mode of infection contributes to the horizontal propagation of CPV to other insects.

Before the replacement of infected gut cells, which happens normally at larval moult (Yamaguchi, 1976), a transmission of virus particles from infected to newly regenerated differentiated cells occurs through the plasma membrane (Kobayashi, 1971). This phenomenon contributes to the viral disease persisting in larvae for a longer time than in lethal infection. Chronically infected larvae act, in this way, as an ideal system for the amplification in nature of a biological insecticide such as CPV. However, resistance of newly formed cells to CPV infection has been reported which resulted in recovery of the insect from disease and in a resistance to a challenge dose of CPV (Yamaguchi, 1979). The response of differentiated regenerated cells to CPV infection varies according to the insect and CPV strain (review of Hukuhara, 1985). This differential viral permissivity of regenerative cells of each insect will determine in some way the degree of CPV virulence as well as recovery rate of larvae from CPV infection (Hukuhara, 1985).

CPV infection is frequently transmitted from one generation to another (Aruga, 1971; Bellemare and Belloncik, 1981; Katagiri, 1981; Sikorowski and Lawrence, 1994). Moreover, CPVs are amongst the most prevalent and persistent pathogens in mass rearing of insect colonies (Aruga, 1971; Belloncik, 1984; Mery and Dulmage, 1975). However, mechanisms of vertical viral transmission are still not fully understood. Females, as well as males,

transmit the viral infection to offspring (Sikorowski and Lawrence, 1994). Polyhedra are detected in CPV-infected adults (Neilson, 1965) and in meconium from these adults (Tanada and Chang, 1960; Sikorowski *et al.*, 1973; Bellemare and Belloncik, 1981). Therefore, the surface of eggs laid by infected females is easily contaminated by CPV polyhedra and this vertical transmission can be prevented with an external disinfection of eggs with chemicals such as formalin (Bullock *et al.*, 1969). However, several other observations and experiments suggest a trans-ovum transmission of CPV (Aruga and Nagashima, 1962; Hukuhara, 1962) which could explain the CPV infections noted frequently by several workers in insect colonies. For example, Grace (1962) noted the induction by an NPV of CPV infection in an ovarian cell culture which indicates that CPV was present in the original ovarian cells in an occluded state. Different stressful situations such as chemical treatments (Aruga, 1971), pathogen surinfection (Tanada *et al.*, 1964), overcrowding (Steinhaus and Dineen, 1959), temperature changes (Hukuhara, 1962), etc. were demonstrated to derepress a latent or occluded CPV infection. Later, other observations in laboratories including our own demonstrated spontaneous appearance of CPV infection in insects and cell cultures following foreign viral infection in the same way as noted by Grace. It is not clear whether virus particles are present at low levels in an insect population or if the viral infection is chronic or latent. The detection of CPV genome and transcripts in ovaries or sperm will be a conclusive demonstration of CPV latency. In any case of equal importance is the fact that several internal and external factors as well as different stresses frequently promote outbreaks of cytoplasmic polyhedrosis virus infections whether the viral infection is latent or the viral inoculum is already present. An identification of these derepressing factors and the status of occluded viruses in an insect are important in the prevention, and induction, of outbreaks of CPV infections in commercially reared insects and insect pest populations respectively.

Factors affecting the development of a CPV outbreak are multiple (see Aruga, 1971 and Payne, 1981). It is important to point out that more than twenty years after the review of Aruga (1971) the mechanisms controlling virus induction or activation are still unknown. It has been shown that an undefined combination of factors which include genetic, as well as environmental and nutritional, stresses acts in the activation process in a very close relationship with the physiology of the insect. It is well documented that larvae subjected to alternating high and low temperatures, chemicals such as formaldehyde, EDTA, disodium salt, or agricultural chemicals, or subjected to nutritional qualitative and quantitative changes of diet will develop CPV infection (Aruga, 1971). Intrahaemocelic injection of distilled water has been demonstrated to act as a stressor for the activation of CPV infection in spruce budworm larvae reared in laboratory (Belloncik *et al.*, 1992). How all these factors interact is a very difficult question to answer.

However, it is quite clear that physiological functions of the insect influence CPV replication more than other viral infection. Therefore, changes, or abnormal alterations, of some physiological functions of insects, including those of the midgut, could interact with genetic influences from larvae and induce, enhance or inhibit manifestation of CPV infections in insect population.

Successful use of cell culture systems (Table 1) that allow the replication of different CPVs has permitted the further investigation of more mechanisms of viral propagation and persistence. Interestingly, peculiar relationships between the CPV and insect cells were obtained when results obtained *in vivo* and *in vitro* were compared. In particular, at a cellular level, no lytic effect of CPV replication is noted in cell cultures (Quiot and Belloncik, 1977; Belloncik *et al.*, 1994a). Infected cell lines could be maintained *in vitro* (Figs 16 and 17) for several months without lysis of the cells. Cells may break *in vivo* only under the pressure of numerous polyhedra. Occasionally, some authors noted excretion, in culture medium of cytoplasmic buds containing virus particles (Quiot *et al.*, 1980a). This was interpreted by these authors as a mechanism by which the infected cells react to the viral infection and reject the virions.

Foci of CPV-infected cells are often noted and, despite the fact that no virus particle is released into the culture medium, viral infection can spread in cell culture monolayers (Belloncik, 1989; Belloncik *et al.*, 1994a) as in the larval midgut (Aruga *et al.*, 1963a). Preliminary electron microscopy observations tend to show that viral infection propagates *in vitro* by passage of non-occluded virus particles through plasma membranes of adjacent cells (Belloncik, unpublished results) as observed in the infected midgut by Kobayashi (1971). Moreover, multiplication of infected cells was demonstrated to contribute to propagation of CPV infection *in vitro* (Belloncik, 1989; Belloncik *et al.*, 1994a). Interestingly, for some CPVs the maintenance of viral infection in a cell line has been proved. Persistence of *Ha* CPV infection for more than 100 passages in an *Ha cell* line was obtained during which time polyhedrin was continuously expressed and polyhedra were formed in the cell system (Belloncik *et al.*, 1994a). Similar mechanisms of persistence of CPV infection were later demonstrated for other virus strains such as *Dendrolimus punctatus* and *Choristoneura fumiferana* CPVs (Belloncik *et al.*, 1992, 1993). However, for other virus strains an *Es* CPV virus infection was observed for only 15 passages of infected cells. This was interpreted as being due to the presence in some infections such as *Ha* CPV, of larger amounts of the non-occluded virions responsible for more efficient and consequently better propagation and persistence of CPV in cell culture (Rocheleau, 1987; Belloncik *et al.*, 1989). This kind of relation between the CPV and the cell is important to consider for mass production, in insect cells, of some CPVs as viral insecticides and expression vectors for foreign genes.

4.6 CYTOPATHOLOGY OF CPV-INFECTED INSECT CELLS

Most common histopathogical changes are easily detected when larvae are dissected. Symptoms of viral infection consist of an enlargement of the midgut which takes on a milky white colour (Fig. 20) owing to the large accumulation of viral inclusion bodies. Iwashita (1971) and Kobayashi (1971) provided extensive information, at a cellular level, on the different alterations noted in epithelial midgut cells. The cytoplasm of cylindrical cells is the first altered by CPV when polyhedra appear in the apical region (Iwashita, 1971). Pathological changes related to the presence of viral polyhedra are normally restricted to cylindrical cells. However, infections have been reported in differentiated regenerative cells of *B. mori* (Iwashita, 1971) as well as goblet cells of *Phalera bucephala* (Iwashita, 1971), *Aglais urticae* (Iwashita, 1971), the douglas fir tussock moth *Hemerocampa (Orgya) pseudotsugata* (Martignoni *et al.*, 1969) and very rarely those of *B. mori* (Saito and Yamaguchi, 1960). When the cytoplasm is filled with numerous polyhedra, the enlarged cells extend into the lumen (Iwashita, 1971). Thereafter infected cells are ruptured and polyhedra are released into the lumen (Fig. 7). Polyhedra are also recovered from infected pupae and adults.

No major change is detected in the nuclei of infected cells despite a possible involvement in viral RNA synthesis (Watanabe, 1966; Hayashi and Retnakaran, 1970). On some occasions hypertrophy and crystal(s) of polyhedrin (Fig. 15) of some *Bm* CPV strains are noted (Hukuhara, 1985). The Golgi complex, microtubules and mitochondria seem not to be affected by CPV infection. However, the abundant endoplasmic reticulum of normal cells is degraded and scattered among the ribosomes, at the end of a CPV infection (Kobayashi, 1971). Lysosome activity has been correlated in midgut cells and *in vitro* cell cultures to an early stage of CPV replication. Moreover, the disappearance of microvilli was noted in CPV-infected midgut cells which may contribute to a decrease in the capacity for nutrient absorption by larvae. In CPV-infected insects reaching the pupal and adult stages and thereafter histopathological changes are noted in the midgut owing to the presence of viral polyhedra (Iwashita, 1971).

Almost the same basic pathological features are noted in cell cultures infected with CPV. No lysis of infected cells is normally associated with viral replication (Quiot and Belloncik, 1977; Belloncik *et al.*, 1989, 1994a) which is a serious obstacle for the design of plaque assays and therefore for the selection and purification of CPV strains. However, when a high titre of virus is used as an inoculum the cytoplasm is packed with large numbers of small polyhedra (Belloncik, 1989). The cells will thereafter break as in infected insects where the lysis of midgut cells occurs mainly when cells are heavily filled with polyhedra. We can conclude therefore that cell destruction is not related specifically to CPV infection but is a consequence of the mechanical pressure on the cell membrane of the numerous dense cytoplasmic polyhedra.

This also explains the excretion in faeces of intact cells filled with polyhedra.

4.7 ALTERATIONS OF INSECT FUNCTIONS

4.7.1 *Metabolic alterations*

The pathophysiology and biochemical aspects of CPV multiplication in larvae have been studied extensively by Watanabe (1971a) and Miyajima and Kawase (1971) by means of several approaches. These authors demonstrated a decrease in the amount of proteins and nucleic acid of CPV-infected haemolymph and midgut cells. However, a precise analysis of the results discussed in the two reviews, suggests that these modifications, and in particular protein decreases, are mostly related to haemolymph, and that no alteration in midgut cells is detected. This aspect will be discussed later. The amounts of most amino acids were decreased in infected insects from 2 to 10 times normal levels, except for glycine and lysine, which increased in the haemolymph of diseased larvae (Kawase, 1965). Increases in uric acid (Watanabe, 1971a) and ammonia (Kawase, 1965) were demonstrated in infected midguts. Furthermore, a reduction of infected midgut pH was noted late in infection, which could be responsible for the expulsion without dissolution of viral polyhedra into environment (Payne, 1981).

Nucleic acid, mostly RNA, synthesis in infected cells was studied by several authors. The rate of DNA synthesis in infected and healthy midgut cells was demonstrated to be similar except at a very late stage (Watanabe, 1967a). However, Kawase and Hayashi (1965) found higher amounts of DNA in midgut and haemolymph of infected insects.

The rate of synthesis of mitochondrial RNA in infected midgut was demonstrated to be lower (Hayashi and Kawase, 1965; Kawase and Kawamori, 1968) together with an increase in the proportion of free nucleotides in CPV-infected midguts (Hayashi and Kawase, 1965; Kawase and Kawamori, 1968). Ribosomal RNA also decreased (Kawase and Kawamori, 1968) and there was a slight increase in the total RNA content of the infected midgut (Kawase and Hayashi, 1965). For an extensive documentation on this subject the reader may consult the reviews of Watanabe (1971a) and Payne (1981).

Although complete normal viral replication occurs in the midgut, the functions of other different organs and tissues may be also affected by CPV infection. The midgut is a very important part of the digestive system and metabolic activities could be altered as a consequence of nutritional deficiency. According to Watanabe (1971a) an increase in amount of uric acid and nitrogen catabolism is noted in the CPV-infected midgut. In addition, some other enzymatic activities such as mitochondrial cytochrome oxidase

and succinic dehydrogenase as well as alkaline phosphatase and esterase activities were demonstrated to be affected but to a lesser extent.

Insect tissue culture systems have contributed to a better comprehension of the pathophysiology events of viral infection in insects. CPV-infected cells *in vitro* are not lysed by viral infection and therefore can be easily subcultured. It was demonstrated that, in contrast to Reoviruses, no shut-off of cellular protein synthesis occurs during CPV replication (Arella *et al.*, 1984). Recent experimentation conducted in our laboratory has proved that CPV-infected cells can be kept viable, without multiplication, for a period of more than 20 months in the same medium. All these observations lead to the confirmation of some previous results obtained by others in larvae, which suggest that the metabolism of cells such as those of the midgut is not affected by CPV infection. Therefore, we may conclude that the alterations of metabolism observed in the whole insect are related to a consequence of nutritional deficiencies owing to pathological alterations of an infected digestive system rather than to be direct effects of CPV infection.

4.7.2 *Physiological alterations*

The consequences of metabolic alterations in insects are numerous physiological perturbations whose intensities are related to doses of viral polyhedra (Ignoffo and Adams, 1966; Bell and Kanavel, 1976; Bellemare and Belloncik, 1981) and to the developmental stage of the treated larvae (Bird, 1969; Bellemare and Belloncik, 1981). Higher virus concentrations combined with early larval stage result in greater effects.

Insects surviving CPV infection have a significantly extended instar duration (Fig. 21; Bird, 1969; Vail *et al.*, 1969; Kurata, 1971; Magnoler, 1974; Bellemare and Belloncik, 1981; Belloncik *et al.*, 1992; Sikorowski and Lawrence, 1994). Additional larval instars (Magnoler, 1974) due most probably to a hormonal imbalance and starvation are noted. Size (Fig. 22) and weight of CPV-infected larvae are less than those of healthy ones (Simmons and Sikorowski, 1973; Bell and Kanavel, 1976; Bellemare and Belloncik, 1981; Belloncik *et al.*, 1992).

CPV-infected pupae are smaller (Fig. 23) than healthy insects (Neilson, 1965; Ignoffo and Adams, 1966; Bird, 1969; Bullock *et al.*, 1970; Vail and Cough, 1970; Simmons and Sikorowski, 1973; Magnoler, 1974; Bell and Kanavel, 1976; Bellemare and Belloncik, 1981). As observed in CPV-infected larvae, the pupae contain less lipid and proteins (Bell and Kanavel, 1977).

Moreover, deformations of infected pupae (Fig. 24) and adults (Fig. 25) from which polyhedra are recovered are commonly observed. Adults have a reduced wing size and difficulty in emerging (Bellemare and Belloncik, 1981; Neilson, 1965; Ignoffo and Adams, 1966; Magnoler, 1974). These malformations are detrimental to insect survival, longevity as well as to

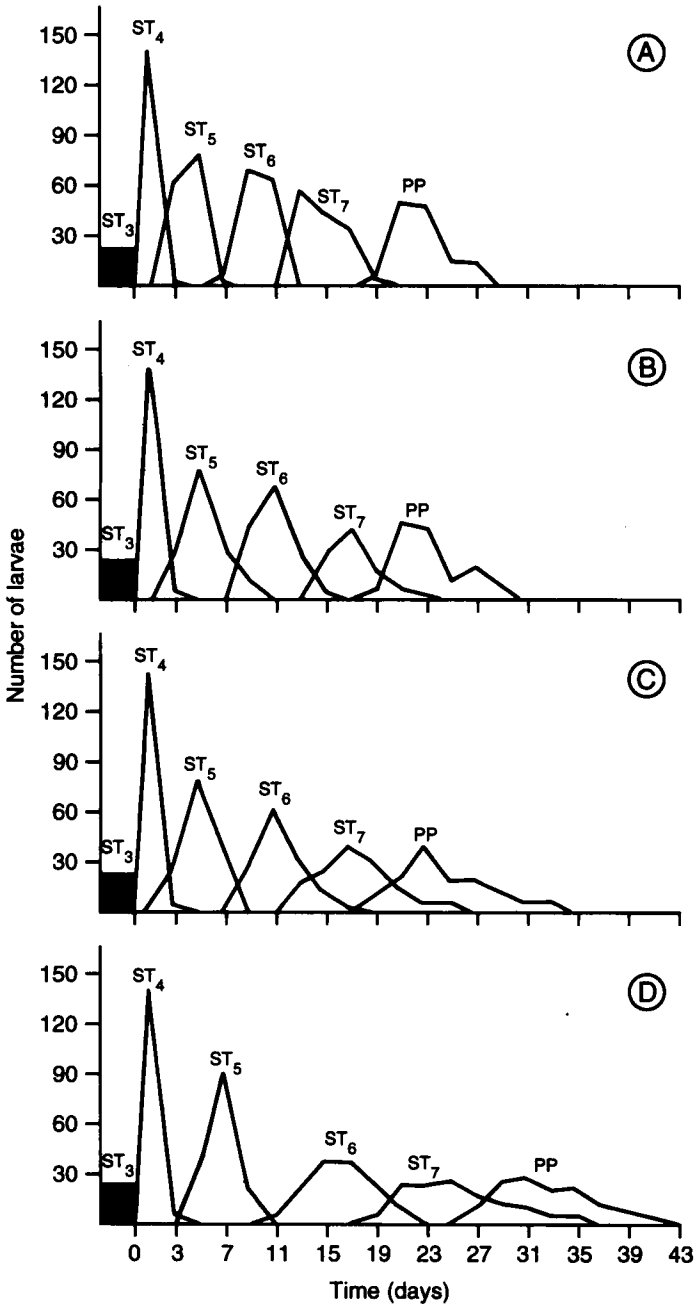

FIG. 21 Delays in development of *E. scandens* larvae infected at the third instar with *Es* CPV. A: Control. B: 10^3 polyhedra per larva. C: 10^5 polyhedra per larva. D: 5×10^6 polyhedra per larva. ST: larval stage (3, 4, 5, 6 and 7). PP: prepupa.

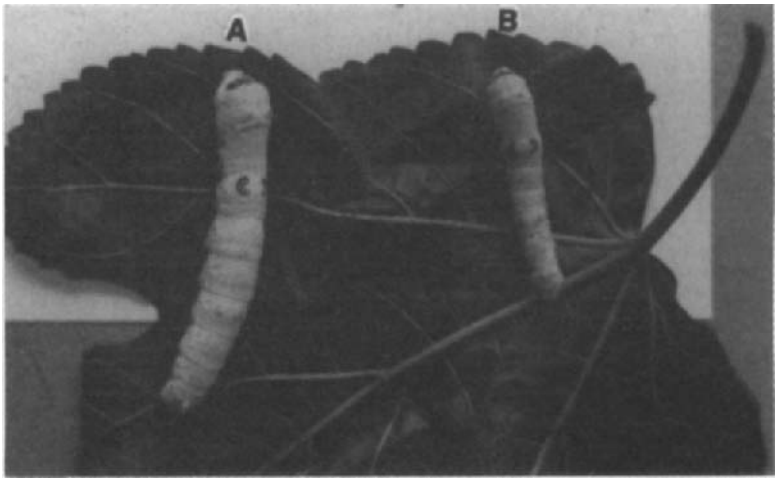

FIG. 22 Detrimental effects of *Es* CPV infection on the size of infected *B. mori* larvae. (A) Control. (B) CPV infected. From Dr S. Miyajima, with permission.

mating ability (Neilson, 1965; Bellemare and Belloncik, 1981), and fecundity of moths.

Infected females, demonstrating poorly developed oocytes (Neilson, 1965), have a highly significant reduced fecundity in relation to their smaller size (Neilson, 1965; Vail and Cough, 1970; Simmons and Sikorowski, 1973; Bonenfant, 1983). The lower number of eggs laid is most probably related to the reduction in size and content of lipids of infected larvae, pupae and adults (Bell and Kanavel, 1977; Sikorowski and Thompson, 1979).

Furthermore, as CPV infection is transmitted usually to the next generation (Aruga, 1971; Bellemare and Belloncik, 1981; Katagiri, 1981; Sikorowski and Lawrence, 1994), the viability of offspring may be affected (Neilson, 1965; Bonenfant, 1983). In this way, the virus infection will have more drastic detrimental physiological and lethal effects on the insect population when insects are first contaminated during early larval development.

The breakdown of lipid reserves of CPV-infected larvae results in an increase in oxygen uptake (Payne, 1981). Moreover larval and adult pigmentation is reduced (Ignoffo and Adams, 1966; Magnoler, 1974) either because of a tyrosine deficiency resulting from less amino acid being available in infected haemolymph or to a hormonal imbalance which affects the tyrosinase activity (Magnoler, 1974; Payne, 1981).

If larval stage durations are extended, one can question the efficacy of CPV applications to reduce the damage from phytophagous pests since infected larvae will consume for a longer period of time. This argument cannot be taken into consideration since CPV-infected larvae become inactive (Arnott

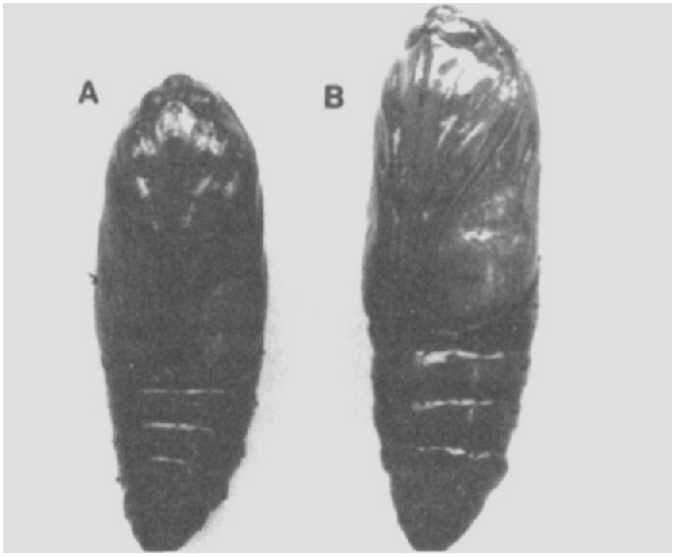

FIG. 23 Detrimental effects of CPV infection of *E. scandens* larvae on the size of surviving pupae. (A) CPV-infected. (B) Control.

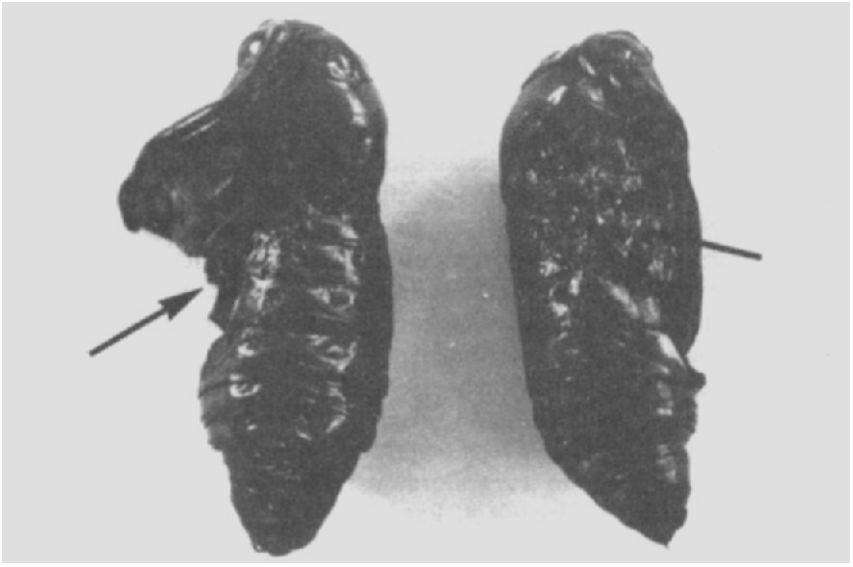

FIG. 24 Deformations of pupae surviving CPV infection.

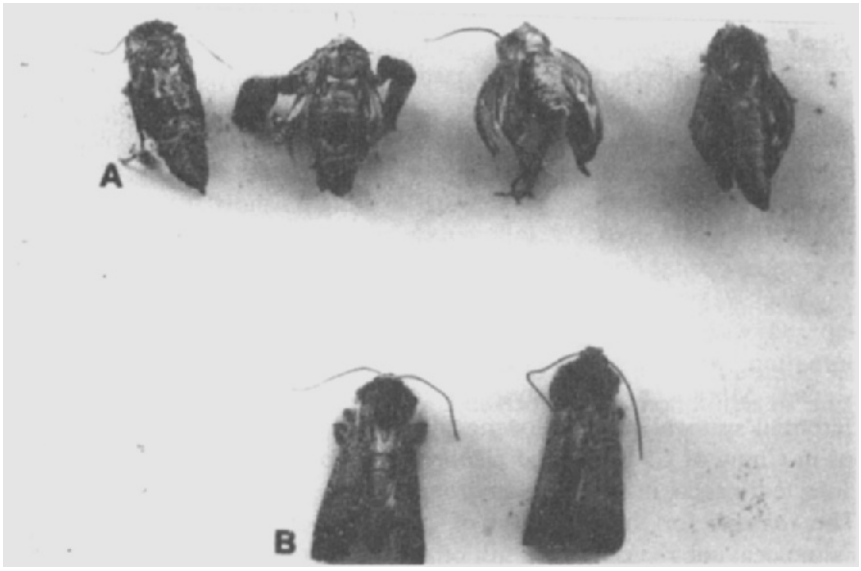

FIG. 25 Detrimental effects of CPV infection of *E. scandens* larvae on surviving adults.

et al., 1968) and cease feeding. In the case of infected *D. plexippus* (Arnott *et al.*, 1968) this change of behaviour happens at around 48 hours of post-CPV infection and the larvae die 10 days later. Experiments conducted in our laboratories demonstrated also, that *Euxoa messoria* larvae infected with *E. scandens* CPV consumed in total significantly less quantity of food even though the duration of their larval period was significantly extended and that nutritional decrease was more severe if CPV was applied at early larval stages. These conclusions were corroborated by field observations such as the reduction, in Africa, of damage to rice caused by *Maliarpha separatella* chronically infected in nature by an endogenous CPV (Pollet, 1980).

5 Reactions of CPV-infected insect cells *in vivo* and *in vitro* to exogenous factors

5.1 SUSCEPTIBILITY TO ENVIRONMENTAL STRESS

Since viral replication induces several metabolic and physiological alterations in surviving insects, it is interesting to investigate the reactions and resistance capacity of CPV-infected insects to detrimental conditions which often occur in nature.

As pointed out above, high temperature has an inhibitory effect on virus replication. In contrast, low temperature permits only partial replication and preservation of infectivity of virus particles in the insect cell (Truong, 1990; Belloncik and Truong, 1991). In addition, low-temperature treatment activates endogenous CPV replication (Hukuhara and Aruga, 1959) and has detrimental effects on the survival of an insect population infected by CPV. In pilot studies, for example, we observed a higher mortality of *E. scandens* larvae treated with *Es* CPV following a rapid and exceptional decrease in temperature during one summer (Belloncik *et al.*, 1994b). In other studies Katagiri (1981) proved that CPV-infected *Dendrolimus spectabilis* larvae, compared with healthy populations, were less resistant in the forest to hibernation, which resulted in a high mortality rate of CPV-treated insects in Japan. Moreover, an analysis of results obtained by Chen (1990) on differential survival rates following hibernation in different climatic conditions in China of CPV-infected *Dendrolimus* could indicate a lower survival of infected insects in a colder environment.

The reasons for this decrease of resistance are unknown. One of the physiological imbalances as a result of CPV infection is an extension of larval stage duration which therefore results in hibernation of larvae at non-favourable stages. It is also well known that freeze-tolerant insects such as *C. fumiferana* adapt physiologically before the cold season and that the capacity of larvae successfully to overwinter is dependent on the nutritional status of the parents (Han and Bauce, 1993). In addition, these insects have haemolymph ice nucleators (Zachariassen and Hammel, 1976), cryoprotectants and/or antifreeze elements (Duman, 1977; Storey and Storey, 1991). Among the common cryoprotectants, the glycerol, converted from stored glycogen (Storey and Storey, 1991), and antifreeze proteins (Hew *et al.*, 1983) are detected in overwintering insects (Lee, 1991; Han and Bauce, 1993). Therefore, it cannot be excluded that physiological and metabolic alterations in larvae owing to CPV infection will affect the synthesis of a cryoprotectant, which would explain the high mortality rates in overwintering CPV-infected insects.

5.2 SUSCEPTIBILITY TO CHEMICALS

Under natural conditions, an insect population is frequently exposed to chemicals and pathogens. Therefore, it is interesting and mandatory when developing biological control programmes to investigate the interrelations of a particular insect disease, such as viral cytoplasmic polyhedrosis, with other pathogen infections of insects. Furthermore, insect colonies are currently used in different laboratories for mass production and for testing of pathogens and chemicals. Considering the lethal, chronic and persistent effects of CPV, it is first very important to diagnose its infection but also to assess its effect on the evolution of various other pathogens and chemicals. Moreover, since CPV infection has been demonstrated to be more chronic and slow in action

rather than lethal but nevertheless effective in the long term, an interest in the formulation of microbial mixtures has been developed. In addition, the interactions in the larvae between CPV and other lethal pathogens have been investigated.

Synergistic interactions between CPV and permethrin, a current chemical insecticide used in pest control, have been demonstrated in the laboratory (Rud and Belloncik, 1984). A higher mortality rate was obtained in CPV-infected insect populations treated with permethrin, and less chemical was needed to achieve the same mortality level if the insect was viral infected (Rud and Belloncik, 1984). This synergistic interaction was demonstrated also when larvae were NPV infected. It will be of interest to determine if CPV infection of larvae predisposes insects to pesticide susceptibility and enhances the effects of other detrimental environmental conditions by means of metabolic and physiological alterations associated with CPV infection.

5.3 SUSCEPTIBILITY TO PATHOGENS

The important question to answer is whether CPV-infected insect populations are more or less susceptible to bacteria, viruses and to other insect pathogens such as fungi or microsporidia. Results published by numerous authors show that the nature of interactions are complicated by several factors. Mixtures of pathogens could lead to synergistic, antagonistic or independent interactions.

The association of CPV with microsporidia, which has similar physiological effects on insects, is not well documented. To our knowledge, the only observation of such a dual infection was made when *Es* CPV was first isolated (Quiot and Belloncik, 1977) along with a microsporidia. Despite the fact that larvae reared in the laboratory were heavily CPV infected, the development of microsporidia in the same CPV-infected cell was noted without, however, an increase in larval mortality rate.

The physiological alterations and mortalities noted when insects are infected by CPV are enhanced significantly when bacteria such as *P. maltophilia*, *B. subtilis*, *E. coli* and *S. epidermidis* are present as contaminant (Bong and Sikorowski, 1991). We have also demonstrated in our laboratory that CPV-infected *C. fumiferana* larvae were more susceptible to bacterial contamination and that synergistic interactions in relation to insect mortality were obtained between CPV and *B. thuringiensis* infections (Belloncik *et al.*, 1996). It is also suggested that endogenous bacteria develop better at the low pH of the midgut, produced as a result of CPV infection, and cause together with CPV infection a larger detrimental effect on larvae (Kodama and Nakasuji, 1969; Hukuhara, 1985). In the same way, Katagiri (1981) and Chen (1990) demonstrated an increased mortality in *Dendrolimus* treated in the forest with a mixture of CPV and *B. thuringiensis* compared with a single pathogen application.

Some types and strains of CPVs are lethal but a cytoplasmic polyhedrosis is mainly chronic, persistent and slow in evolution. In contrast, NPV is more commonly lethal. A combination of the two pathogens will therefore have some interesting practical applications in both the short- and long-term biological control of insects. Different observations in experimental fields demonstrated a contamination of NPV with CPV together with a cohabitation of the two viruses in natural *P. unipunctata* populations over many years with, however, predominance of CPV (Tanada, 1966). Other epidemiological studies demonstrated over several years the occurrence of the two viruses in *Lymantria fumida*. Some laboratory studies have been conducted on the relations between CPV and NPV in different insects such as *Pyrausta cardui* (Smith and Xeros, 1953), *Choristoneura fumiferana* and *Malacosoma disstria* (Bird, 1969). Several conclusions have been drawn on the effects of CPV contaminating an NPV inoculum, but no clear feature of the interactions has been presented. Interference as well as synergetic interactions have been demonstrated. It is important to point out that several parameters, such as the sequence of applications, the ratio of the two viruses, and larval development, influence the nature of the interactions. Several observations (Piasecka-Serafin, 1977; Cunningham, personal communication) indicate that contamination of NPV with CPV increases the efficacy and lethality of NPV. Moreover, studies conducted in our laboratory demonstrated that lethality and rapidity of action of *Agrotis segetum* NPV were increased significantly if *E. scandens* larvae were previously infected with CPV. Lethality half-times, as well as mortality rates, of larvae were significantly improved following these dual treatments (St-Amand, 1982; Belloncik *et al.*, 1982).

Recently a simultaneous infection of *B. mori* cell lines with *Es* CPV and *Bm* NPV has been achieved but CPV and NPV were not observed in the same cell (unpublished observations). However, Quiot *et al.* (1980b) obtained a dual infection of *Es* CPV and *Galleria mellonella* NPV and demonstrated the replication of the two viruses in a single cell. Interference between the two viruses was noted during the process of baculovirion inclusion in polyhedra and the synthesis of the NPV particle membrane. Hypersynthesis and crystallization of polyhedrin, which characterizes the *Es* CPV strain, were suspected as cause of this interference. When the *Es* CPV polyhedrin was expressed in the nucleus using a baculovirus expression system, no negative effect of the replication of the baculovirus on the expression of CPV polyhedrin and the maturation of polyhedra was observed. However, the use of the baculovirus as expression vector for the CPV polyhedrin gene changes the location of the *Es* CPV polyhedra from the cytoplasm to the nucleus. It was also suggested that an interferon-like substance may be produced by CPV which interferes with baculoviral replication (Quiot *et al.*, 1980b). If such phenomenon occurs, it will affect NPV replication *in vivo*. We noted, however, synergistic interaction in larvae (St-Amand, 1982; Belloncik *et al.*, 1982).

The effect on CPV replication of another DNA virus has also been studied. No synergism or interference was noted when larvae of *E. scandens* were infected with *Chilo* iridescent virus (CIV) and *Es* CPV (unpublished results). However, interestingly, significant enhancement of the infection rate and the number of polyhedra of *Es* CPV was obtained when these two viruses replicated together in the cytoplasm of *C. fumiferana* 124 cell line (Arella *et al.*, 1984). The enhancement was induced by the replication and not by the components of the cytoplasmic DNA virus. The nature of synergitical interaction between the CIV and the CPV infected cells has not yet been elucidated and merits further investigation.

NPV and CIV are not normally found in midgut cells, whereas complete replication of CPV is restricted to these cells only. Therefore, in contrast to *in vitro* replication, the co-replication of CPV and NPV or CIV in one insect cell is an unlikely possibility. The conflicting results obtained *in vivo* and *in vitro* could be related to differential cellular tropisms. Interactions between CPV and the two other DNA viruses occur at a cellular level rather than by synthesis of interferon-like or synergistic substances. Direct interactions between the replication of the two viruses or cell modifications associated with the replication of one virus will result in synergistic or antagonistic interactions. However, in larvae, the CPV and NPV or the CPV and CIV will interact with each other via several physiological and metabolic alterations specific to their infection, which could result in significant decreases of DL_{50} and LT_{50} values.

6 Practical considerations

Compared with other insect viruses, several peculiar characteristics of CPVs reflect their important potential for applications in biological control programmes of pests, as well as insect in physiology and genetic engineering fields.

6.1 CPVs AS CONTAMINANTS

Several personal communications and reports indicate frequent CPV contaminations in mass-reared insect colonies such as *E. scandens*, *E. messoria*, *B. mori*, *H. armigera*, *C. fumiferana*, *L. dispar* and *H. virescens*. Persistent and debilitating effects on insects owing to CPV infection result in drastic decline of insect colonies. Insect physiologists and pathologists must also be aware that CPV, in frequent occluded or active forms, affects insect physiology and therefore significantly alters the results obtained in their respective experiments.

Despite the progress being made towards a better comprehension of virus biology and replication, and several tentative suggestions for insect protection

with inactivated viruses and treatments of insects with chemicals and high temperatures, no efficient prophylactic procedure is yet available to prevent an insect colony from CPV infection. Until the time when the exact mechanism of vertical transmission of CPV is known, and therefore controlled, disinfection of eggs and all other rearing equipment with chemicals such as formalin and hypochloride solutions (Sikorowski and Goodwin, 1985) is the only way to prevent and control the further propagation of a CPV infection in mass-reared colonies. However, it is also important to point out that maintaining healthy insect colonies by providing optimal nutritional and environmental conditions will also be an important step towards the control of the evolution of a CPV infection.

6.2 CPVs AS BIOLOGICAL CONTROL AGENTS OF INSECT PESTS

Cytoplasmic polyhedrosis viruses have received, in the past, less interest than baculoviruses as biological control agents. The main reasons are their lower lethality to larvae and the slower action of CPVs compared with nuclear polyhedrosis viruses. Another reason, for safety considerations, is their taxonomic relatedness to other vertebrate *Reoviridae*. However, as has been shown in this chapter, CPVs are very infectious, persistent and cause high larval mortality rates in synergy with various exogenous natural factors such as bacterial contaminants and pathogens, climatic conditions and chemicals. Metabolic and physiological alterations in surviving insects induced by CPV infection cause a decrease of larval consumption combined with an overall high mortality over an entire generation of insects.

In general, it is clear that CPV will be more useful for plant protection in stable ecosystems such as forests and when a certain level of damage can be tolerated (Payne, 1981). Examples of good control of insects with CPV, cited in the review of Granados (1978), include those of *Lymantria dispar* and *fumida* in Japan, *Thaumetopoea pityocampa* in France and *Trichoplusia ni* in USA with their respective CPV. In addition to this list, CPVs of *Dendrolimus pini* against *D. pini* and *Malacosoma neustria* showed promising control actions. Up to now only the CPV of *Dendrolimus spectabilis* has been commercialized in Japan as Matsukemin product (Aizawa, 1976; Katagiri, 1981). Efficacy of this virus alone, or synergistically with *Bt*, has been demonstrated in Japanese forests (Katagiri and Iwata, 1976; Katagiri *et al.*, 1977). Moreover, in China, CPV together with *Bt* has been used for more than twenty years on a large scale against *Dendrolimus* with satisfactory results (Ying, 1970; Hsiao, 1981; Chen, 1990). In Canada, despite the efficacy of spruce budworm and forest tent caterpillar CPVs suggesting their possible use in biological control (Bird, 1969), no field utilization of these viruses has been made. However, recent work on *C. fumiferana* CPV has demonstrated the efficacy of this CPV (Belloncik *et al.*, 1992) alone or in synergy with *Bt*

(Belloncik *et al.*, 1994). Therefore, the CPV of spruce budworm could in future be another example of the importance of CPV as a viral insecticide in forests.

Taxonomic relatedness of CPVs to vertebrate viruses, such as reoviruses, could represent a handicap for their use as viral insecticides. However, the results of extensive biosafety testing of CPVs, conducted in several laboratories, on different vertebrate hosts including mice, rabbits, avian embryos and fish, as well as on vertebrate and mosquito cell lines, demonstrated no pathogenicity and replication of CPVs for non-target organisms (Cantwell *et al.*, 1968; Granados, 1975; Katagiri, 1981; Belloncik *et al.*, 1986b, 1987; Chen, 1990). Moreover, no viral RNA was detected in any vertebrate tissues tested either *in vivo* or *in vitro* (Belloncik *et al.*, 1986b, 1987).

In conclusion, several demonstrations of the effectiveness of CPVs combined with results of biosafety studies and the development of cell culture systems for the production of CPVs using conventional medium (Belloncik *et al.*, 1989, 1993) or semi-defined serum-free medium (Belloncik and Akoury, 1993), contribute to a renewed interest in the use of these viruses as viral insecticides.

6.3 CPVs AS EXPRESSION VECTOR OF FOREIGN GENES

The hypersynthesis phenomenon of viral coded polyhedrin in cells infected with some insect viruses has been over the last ten years the basis for the development of useful vectors for high-level expression of several foreign genes. Among the virus candidates, only the baculoviruses were extensively used for the production in insect cell culture (Luckow and Summers, 1988; Miller, 1988) and more efficiently in silkworm larva systems (Maeda *et al.*, 1985; Kobayashi *et al.*, 1992), of several medically and biologically important recombinant proteins. However, baculoviruses are highly and rapidly lethal for larvae as well as cytolitic, which represents a limitation to the quantity of expressed products. CPVs have advantages over baculoviruses as expression vectors of foreign genes since CPV infection is not lytic to cells, and would not be lethal for larvae. Consequently, more expressed protein may be obtained during an extended infection period. Moreover, amplification cycles and the persistence *in vivo* and *in vitro* (Belloncik *et al.*, 1994a) of CPV infection are another advantage over lethal baculovirus and other insect virus infections. Therefore, the use of cytoplasmic polyhedrosis viruses as non-cytolytic expression vectors is attractive. However, three difficulties related to CPV replication and composition are encountered and must be resolved. First, it is not known if foreign genes could be easily incorporated into the CPV particle and genome. Second, while the 10 segments of ds RNA are easily transfected into insect cells (Kobayashi and Belloncik, 1993), the

transfected viral ds RNA is not infectious. Some of the new methodological approaches used for the transfection of ds RNA which have resulted in the successful replication of reoviruses (Roner *et al.*, 1990) must be adapted to CPV. Third, no plaque assay is available for CPV cloning and the selection of viral recombinants lacking the polyhedrin gene is therefore difficult to achieve.

7 Conclusion

Historically, CPVs were first studied as noxious micro-organisms and contaminants of insect colonies. However, several characteristics of these viruses, treated in this chapter, have combined to increase their importance in a range of applied and fundamental research programmes.

Cytoplasmic polyhedrosis viruses have interesting particularities compared with other insect viruses in use or being considered as candidates in biological control programmes of insects. These particularities are based on interesting relationships that develop both *in vivo* and *in vitro* between the insect host and virus infection. The numerous physiological and metabolic disturbances produced as a consequence of CPV infection can act as important potentiators and enhancers of insect mortalities. This, combined with the persistence of viral infection throughout insect generations, must be exploited more in the development of biopesticides.

This chapter has also reviewed the complex interrelations, during the evolution of CPV infection, between CPV strains and different endogenous as well as exogenous factors for an insect cell host *in vivo* and *in vitro*. These types of interrelations are typical of several other microbial infections of insects but also of vertebrates. Therefore, our knowledge of evolutionary patterns of CPV infection may help us to understand better the evolution of several other microbial pathogeneses, including those caused by HIV, where complex interrelations between virus infection and different endogenous and exogenous factors play a preponderant role in the evolution of viral infection and pathogenesis.

It should also be borne in mind that the discoveries of important biological and molecular phenomena, such as the capping of eukaryotic mRNA, were made during research on one CPV. Thus studies conducted during the last three decades on the replication of and infection by cytoplasmic polyhedrosis viruses are important not only for the biological control of insects but also for insect physiology, molecular and cellular biology, comparative virology, and microbial disease, as well as genetic engineering disciplines. As a consequence of this importance, a renewed interest in CPVs has been recently noted and the number of laboratories in the world involved in research on cytoplasmic polyhedrosis viruses is increasing. It is expected and hoped that this interest will continue to intensify in the future.

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Interaction of Circulation and Tracheal Ventilation in Holometabolous Insects

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1 Introduction

In comparison with Crustacea and Chelicerata the haemolymph vessels of the open circulatory system in insects are further reduced in most orders to the single dorsal vessel. This is differentiated in most adults, and more rarely in larvae, into an abdominal heart tube and a thoraco-cephalic aorta. Haemolymph transport, especially in adults, is aided by the ventral diaphragm in some insect orders and by accessory pulsatile organs, situated in the head at the antennal bases, in the legs, in the pterothorax and near the abdominal processes, often without a direct morphological connection with the heart (Jones, 1977; Miller, 1985; Krenn and Pass, 1994, 1995; Pass, in press).

The direct supply of tissues with atmospheric air by a ramifying tracheal system relieved the haemolymph of oxygen transport and led to the loss of respiratory pigments. According to the manyfold insect constructions, the open haemolymph system and tracheal system show extremely different morphological and functional adaptations. Even in the course of the lifetime of a single species, a large variety of circulatory and respiratory constellations may be realized. This refers especially to holometabolous insects. Our knowledge of the circulatory system has recently been reviewed by Jones (1977), Noirot and Noirot-Timothée (1982) and Miller (1985). The current knowledge about the respiratory system, mainly of the hemimetabolous insects, has been reviewed by Miller (1974, 1981) and Mill (1985). The present article will focus on the changes of the haemolymph- and tracheal system during development of the most successful flight-adapted holometabolous insect groups.

2 Circulation and respiratory gas exchange in larvae

Larvae are adapted to a more or less phlegmatic sitting and feeding way of life with the necessity to store fat. At the end of their growth, they gain a higher body weight than the adults (Reinecke *et al.*, 1980; Seifert *et al.*, 1989; Fig. 1). They contain a relatively high haemolymph volume (15.2–20% of their body weight in *Celerio* (Heller and Sweichowska, 1948) and 34% in *Acherontia atrapos* (L. T. Wasserthal, unpublished, Fig. 1). However, if one considers that about 40% of their body weight consists of intestinal contents as in *Manduca* (Reynolds *et al.*, 1985), the haemolymph volume related to the weight of the living tissues is as high as 45–60%. The haemolymph is continuously pumped by the heart from the rear to the head (Gerould, 1933, 1938; Jones, 1954; L. T. Wasserthal, unpublished; Fig. 2a–b) and flows backward through the uniform haemocoel.

The tracheal system consists of ramified and anastomosing tubes with

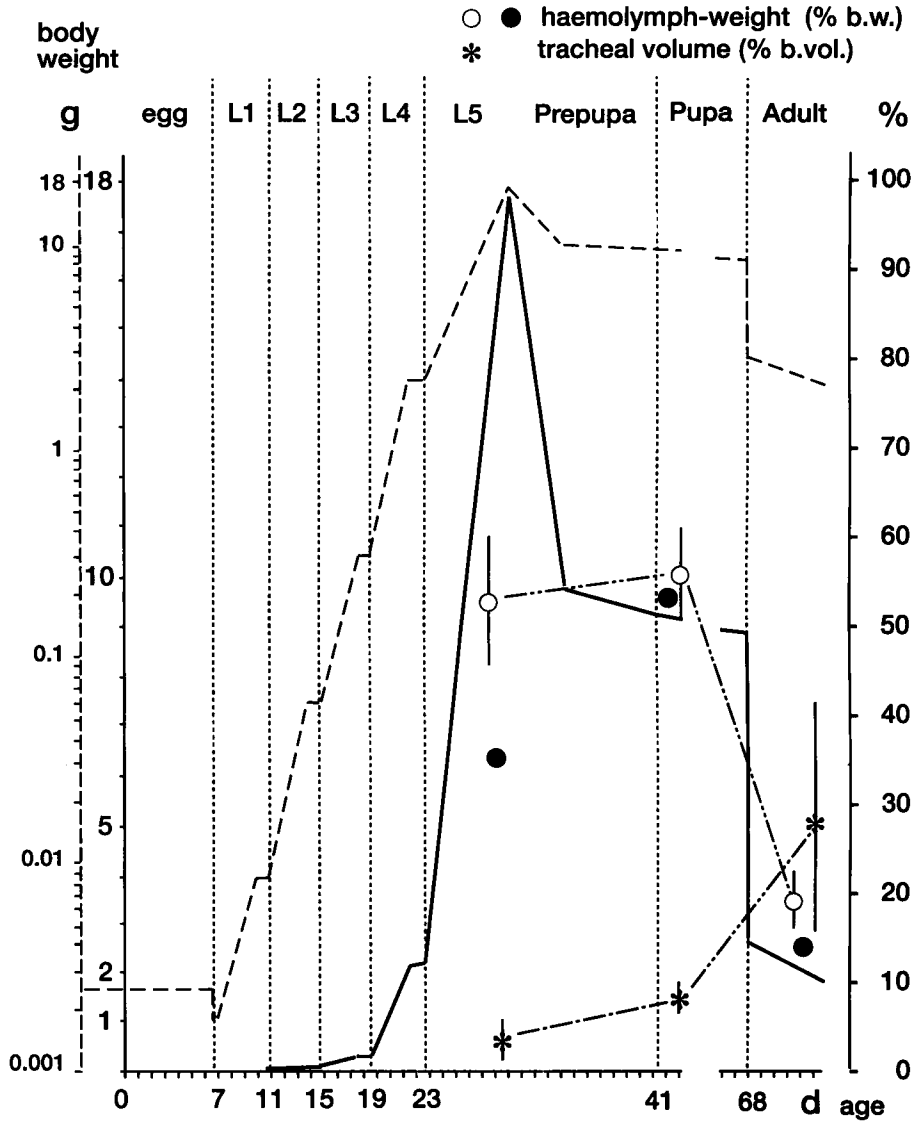

FIG. 1 Changes in body weight (g), haemolymph content (% b.w.) and trachael volume (% b.vol.) during ontogeny of the hawkmoth *Acherontia atropos* L1–L5 = larval instars 1–5. ○ = haemolymph-content (exsanguination-method), including contents of intestine, ● = haemolymph-content (exsanguination-method), contents of intestine subtracted. * = tracheal volume (L. T. Wasserthal and P. Rauch, unpublished). The haemolymph- and tracheal parameters are given for the last instar larva at the switch to the migration phase, for a 10-day-old or diapausing pupa and for the 2–3-day-old moth before first honey uptake.

generally simple coiled taenidia. The respiratory gas exchange in soft-bodied larvae is said to be by diffusion alone, since deformations of the body wall do not lead to measurable tracheal pressure changes (Krogh, 1920). This can be explained with the elastic integument and with the construction of tracheae which are stabilized by massive spiral taenidia combined with a mostly round shape (Fig. 5a), which should largely be resistant to compression. The relatively high form constancy of the larval tracheae is necessary to prevent their collapsing in a haemolymph system with pressure generally above atmospheric. In spinning caterpillars of the wax moth *Galleria* haemocoelic pressure is +100 to +600 Pa (Slama, 1984). In crawling caterpillars irregular peaks in internal pressure can rise up to as much as 2000 Pa. This pressure is not only caused by the interaction of the relatively high haemolymph content with integumental muscle tension, but also by the turgid gut. If larvae are wounded, they often tend to bleed by increasing their haemolymph-pressure via contraction of the integumental muscles. This may be the reason why almost no invasive measurements of haemolymph parameters exist from this instar.

Special ventilatory movements have only been described so far in some specialized dipteran larvae. In aquatic syrphid larvae, two dorsal tracheal sacs are rhythmically compressed to a flat band by contraction of the integumental muscles; the air filling is said to be passive by taenidial elasticity (Alsterberg, 1934). Deflation of tracheae during asphyxis has been described in *Culex* larvae (Babak, 1912). The model of ventilation by active compression and passive relaxation combined with inspiration has often been generalized in textbooks to be of the normal type in insects (Weber, 1933; Mill, 1985). Taenidium-stabilized tracheae can be compressed only in those insects with oval (not round) shaped tracheae such as in fleas (Herford, 1938). There is some experimental evidence that inspiration is more frequent through the anterior spiracles and CO_2 discharge is mainly through the 2 or 3 posterior ones (Wrede, 1926). In caterpillars of giant silk moths and hawkmoths the CO_2 output is continuous if the sum of the outflow from all spiracles is measured with the URAS (ultrared absorbing gas analyser); when the output of single posterior spiracles is measured in *Acherontia atropos* caterpillars, long phases (1–7 min) with a high, rather constant, level of CO_2 outflow alternate with equally long phases with little or no CO_2 output (L. T. Wasserthal and T. Fincke, unpublished; Fig. 2c). Although all segmental tracheal trunks with their branches are interconnected by longitudinal stems, the areas of each segment may be mainly supplied locally via the nearest spiracle. The fact that in hibernating arctiid caterpillars CO_2 output is discontinuous (Punt, 1950) shows that also in caterpillars coordinated spiracular closing is possible under special conditions. Tight spiracular closing also enables survival during hours of submersion in water as in caterpillars of *Parnassius phoebus* and of hawkmoths such as *Acherontia* (L. T. Wasserthal, unpublished).

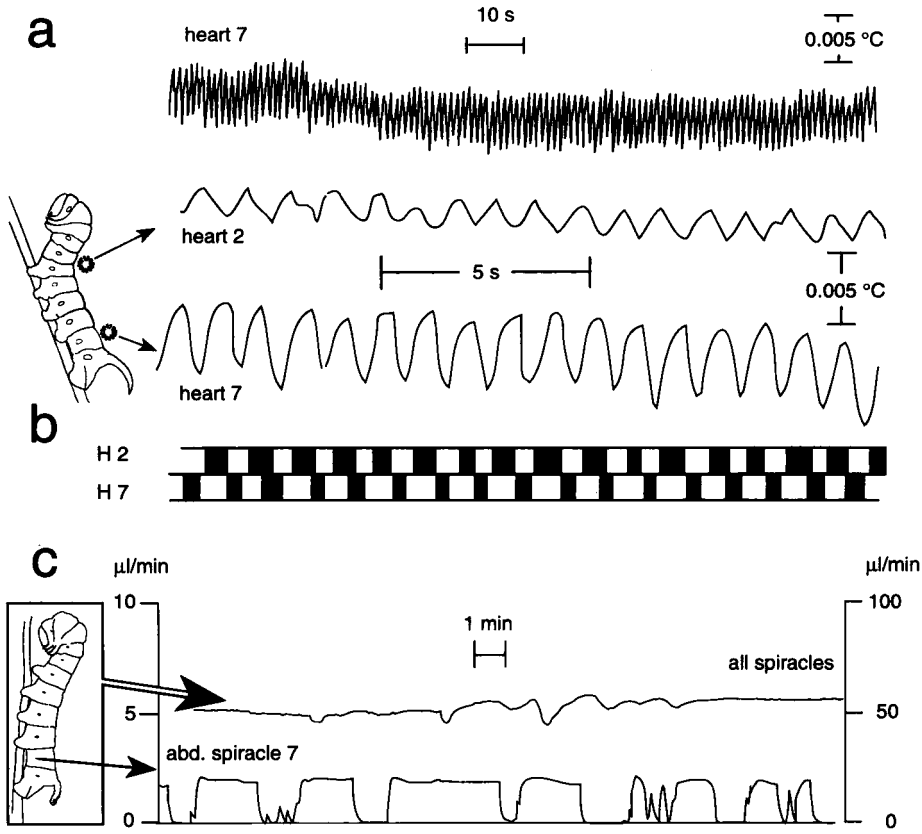

FIG. 2 (a–c) Heart activity and CO_2 emission in last instar (L5-) caterpillars of hawkmoths. (a) Stereotypic forward heart pulsations of a contact thermographic measurement lasting several hours in *Sphinx ligustri* (T_a 23°C). (b) Simultaneous recording of anterior heart (thermistor at 2nd abdominal tergite) and posterior heart (7th abdominal tergite) with higher recording speed showing the metachrony of pulses. (Black bars = convective cooling.) (c) CO_2 emission in *Acherontia atropos*. Upper trace shows continuous CO_2 emission, when the sum output of all spiracles is measured. Lower trace shows discontinuous CO_2-discharge of a single posterior spiracle. CO_2 emission was measured with an infrared gas analyser (URAS); L5, 14 g at T_a 25°C.

3 Circulation and tracheal gas exchange in pupae

3.1 CIRCULATION AND HAEMOCOELIC PRESSURE IN PUPAE

In contrast to the larva, the stationary pupa has a largely sclerotized exoskeleton. It is generally freed from the job of moving and does not have to be turgid. In pupae the haemolymph volume is comparable to that of larvae –

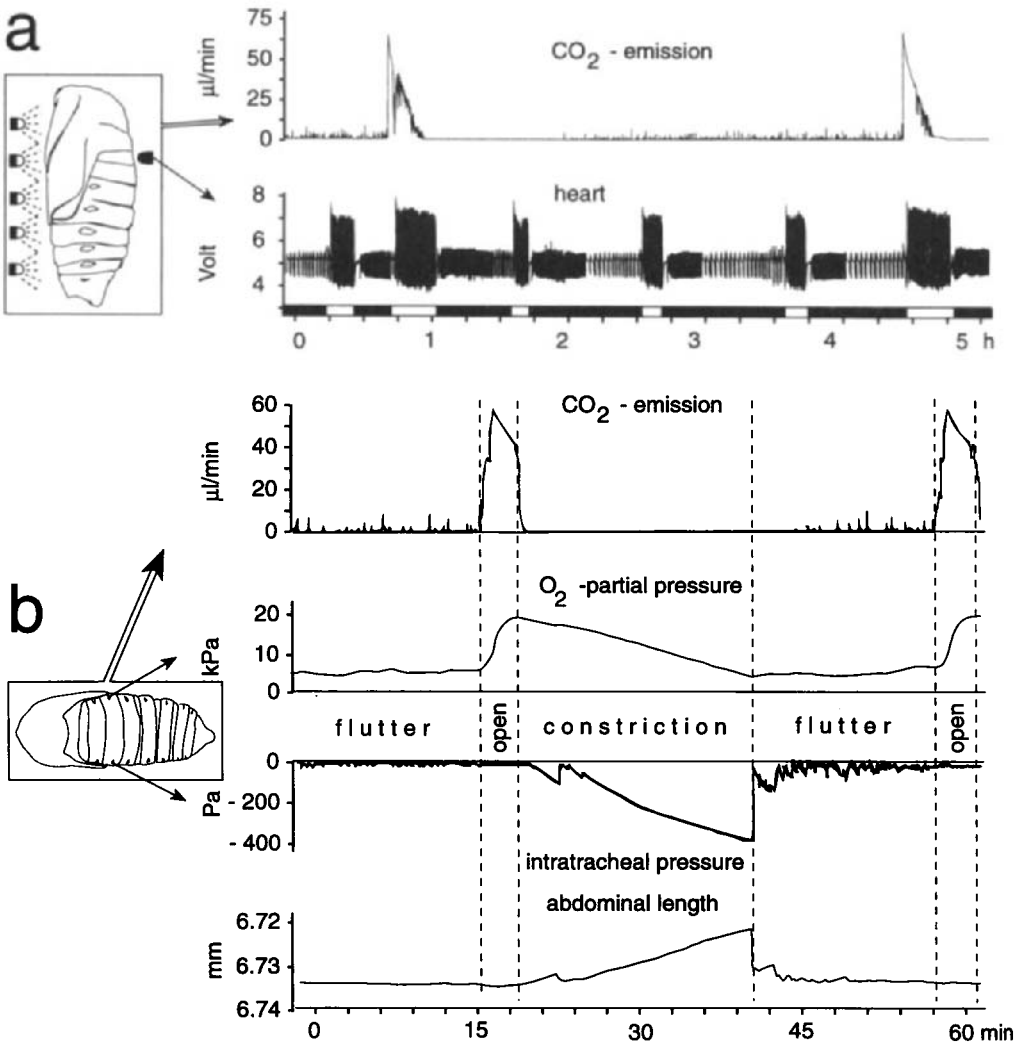

FIG. 3 (a–b) Discontinuous respiratory gas exchange and heartbeat-reversals in diapausing *Attacus* pupae. (a) CO_2 bursts coincide with prolonged forward pulse periods, but heartbeat reversals occur also during the phases of spiracular constriction and fluttering, which may last several hours. CO_2 emission was recorded with URAS. Heart pulses were recorded by an infrared-sensitive diode, which responded to the changing infrared light transmission of the beating heart. The pupa was irradiated from the ventral side by IR-emitting diodes. The forward pulse periods are shorter but have a higher pulse frequency and amplitude than the backward pulse periods (black bars in the signature). Backward pulse periods are frequently interrupted by heartbeat pauses. *A. atlas* (female, 7 g at 20°C, about 5 months after pupation and 5.5 months before eclosion. (L. T. Wasserthal *et al.*, unpublished results). (b)

18.3–42.6% of the body weight in *Celerio* (Heller and Sweichowska, 1948), 53% in diapausing *Acherontia*, where the contents of the intestine, about 3–5% of body weight, are negligible (L. T. Wasserthal, unpublished; Fig. 1). The fluidy consistence of the re-embryonalized interior of pupae is a well-known phenomenon. The heart in pupae is generally subjected to metamorphosis. The aortal region and the posterior end with the alary muscles especially undergo histolysis and are remodelled (Weismann, 1864; Kowalevsky, 1886; Vaney, 1902; Jensen, 1973). The reduction of the posterior heart segments with the loss of the dominant larval pacemaker may explain the long phases of weak, sometimes erratic heart activity with long pauses of hours (Jones, 1977) or prolonged periods of backward pulsations frequently interrupted by heartbeat pauses (L. T. Wasserthal *et al.*, unpublished). Before and after the fundamental changes of the heart organization, it exhibits periodic heartbeat reversals in prepupae and pupae. This phenomenon was described for the first time in *Bombyx mori* pupae (Malpighi, 1669). Later it was observed also in pupae of this species and of other Lepidoptera (Gerould, 1929a,b) and Culicidae (Jones, 1954). The heartbeat reversals may occur more frequently than the periodic CO_2 bursts as in diapausing Saturniidae and Sphingidae (Fig. 3a) or they may be coordinated with discontinuous ventilation cycles as within the initial half of the development of subitan pupae of the papilionid *Troides rhadamantus* (Hetz and Wasserthal, 1993; Fig. 4). In these Lepidoptera the bursts coincide most frequently with the forward pulsations (Figs 3a, 4). Often the heart can be observed to switch from backward to prolonged forward pulse periods during the first seconds of the burst. When the forward pulses have a higher pulse frequency (in *Attacus* pupae: 13.6 ± 3.3/min) and amplitude than the backward pulses (in *Attacus* 8.7 ± 2.1/min, at T_a 20°C, $N = 36$, $n = 97$), they may distribute the haemolymph more efficiently for optimal discharge of the CO_2 from the haemolymph into the tracheal system. But there are also long phases with pulse rate and amplitude not differing significantly between forward and backward periods. Heart activity patterns frequently change in the course of development, and it remains difficult to decide which effects are of functional significance and which are only expressions of the incomplete differentiation. As the anterior and posterior body in this instar are still united by a wide haemocoelic connection and the pulse frequency is very low, a build-up of a lasting pressure decrement is certainly not possible. The

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Simultaneous measurements of 4 respiratory parameters during discontinuous ventilation in *A. lorquinii* pupa (3 months and 20 days before eclosion, female, 7.3 g at 20°C). Intratracheal pressure becomes subatmospheric during constriction of the spiracles due to O_2 consumption visible in the O_2-partial pressure curve. The abdomen passively shortens synchronously and proportionally to the decreasing intratracheal pressure. (S. K. Hetz and L. T. Wasserthal, unpublished). The curves confirm the classic diagram of diapausing *Hyalophora* (Levy and Schneiderman, 1966c).

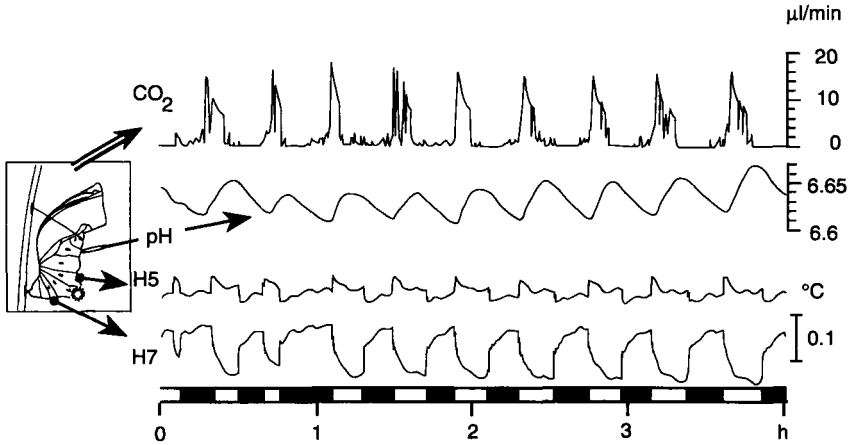

FIG. 4 Heartbeat reversals coordinated with CO_2-bursts and pH-changes in the haemolymph of a 4-day-old non-diapausing pupa of the papilionid butterfly *Troides rhadamantus*. Heartbeat direction was determined by thermistors upon the 5th and 7th abdominal tergites while a heat-marking resistor was arranged between them upon the 6th tergite. The beginning of the forward pulse period coincides with the onset of the CO_2 burst. The pH becomes more acid during constriction and flutter phase of the spiracles, reflecting CO_2 storage in the haemolymph. Male, 5.44 g, at T_a 20°C (Hetz and Wasserthal, 1993 and unpublished).

haemolymph exchange through the waist is probably slower than in caterpillars. In the lepidopteran pupae, the ventral diaphragm with its backward pumping activity is not yet functioning. The heart alone serves for supply of haemolymph to the anterior and posterior regions of the body. During forward pulses haemolymph circulates within the entire body, entering in the abdominal ostia and leaving the aorta in the head. During backward pulses, haemolymph aspiration may switch from the aortal openings to the anterior abdominal ostia before the accessory tergal organs are developed. This may result in a short circuit circulation within the abdomen.

Haemocoelic pressure in pupae is in general continuously subatmospheric. In *Actias gnoma* the negative haemocoelic pressure is between −210 to −255 Pa; in *Saturnia pyri* it varies between −250 to −600 Pa but may briefly increase to +600 Pa by abdominal twisting movements (Slama, 1991). Further different abdominal movements have been described in pupae (Brockway and Schneiderman, 1967; Slama, 1991), many of which seem to be reactions to the disturbance of the experimental situation and seem not to be a regular component of circulation or tracheal ventilation at rest. In small pupae of *Galleria mellonella* haemocoelic pressure is −350 to −450 Pa and in *Pieris brassicae* may reach −350 to −1150 Pa (Slama, 1984). In the puparia of the fleshfly *Sarcophaga bullata* a subatmospheric pressure of −40 to −85 Pa has been recorded. In pupae of the beetles *Tenebrio molitor* and coccinellid

species a negative pressure of about −250 to −600 Pa has been measured (Slama, 1984, 1986).

3.2 TRACHEAL VENTILATION BY 'ABDOMINAL PRESSURE PULSES' IN PUPAE LIBERAE AND 'SUCTION VENTILATION' IN PUPAE OBTECTAE

Pupae have developed a respiratory mechanism specifically adapted to their needs to conserve water and energy, which during pupal life can generally not be supplied from outside. Most pupae and puparia examined as yet show discontinuous tracheal gas exchange. Continuous gas exchange has been described only from diapausing sawfly pupae, living under humid subterranean conditions (Slama, 1960). In coleopteran and honey bee pupae bouts of active abdominal ventilatory movements have been described (Slama, 1976, 1984, 1986; Slama and Rembold, 1987). These 'extracardiac pulses' increase the haemocoelic pressure from −550 to −250 Pa in Coccinellids, from −600 to −300 Pa in *Tenebrio molitor* and from a minimal −20 Pa to a maximal +50 Pa in *Apis*. The pupae liberae of Hymenoptera and Coleoptera are normally only weakly sclerotized. Especially in beetles, the pupal phase is relatively short and does generally not serve as a diapausing instar. This may be the reason why these pupae can afford energetically unfavourable active ventilatory movements. In long-living diapausing puparia with uncompliant sclerotized cuticle such as in the fleshfly, *Sarcophaga*, intermittent O_2 consumption has been recorded (Denlinger *et al.*, 1972). It must be assumed that this intermittent O_2 uptake must be regulated by periodic opening and closing of the spiracles without pumping by abdominal muscles similar to the case in lepidopteran pupae.

The best analysed pupal respiratory system is the 'passive suction ventilation' mechanism in diapausing lepidopteran pupae, especially those of Saturniidae (Schneiderman and Williams, 1955; Levy and Schneiderman, 1958, 1966a–c; Buck, 1962). The tracheal gas exchange is regulated by the opening and closing activity of the spiracles (Fig. 3b). During hermetical closing of the spiracles oxygen is consumed and the intratracheal pressure decreases from atmospheric $P\,O_2$ to a $P\,O_2 = 3.5$ kPa in the tracheal system. In the tracheae a subatmospheric pressure of −350 to −526 Pa is built up. At the concentration level of a $P\,O_2 = 3.5$ kPa, the spiracles begin to flutter, allowing the atmospheric air to enter briefly. Although the main vacuum is abolished after the first flutters, $P\,O_2$ remains low at about 3.5 to 5 kPa as if a critical concentration determined in some way the threshold of the spiracular fluttering (Burkett and Schneiderman, 1974; Hetz *et al.*, 1994). Between each flutter event (microcycle) a slight vacuum arises (Levy and Schneiderman, 1966c; Hetz *et al.*, 1993, 1994; Fig. 3b). It is assumed that during the flutter phase inward convection of air prevents outward streaming of water vapour from the tracheae (Hazelhoff, 1926; Buck, 1962; Levy and Schneiderman, 1966a; Kestler, 1980, 1984). There is, however, during the

flutter phase also little CO_2 outflow from the spiracles (Levy and Schneiderman, 1966a; Hetz *et al.*, 1993; Fig. 3b). The constriction phase is generally much shorter than the flutter phase. One might therefore assume that it serves mainly to build up the diffusive gradient of $P\,O_2$ (3.5–5% intratracheal versus 20% in the atmosphere) which in combination with the slight subatmospheric pressure, arising in the microcycles of the flutter phase, is utilized for combined O_2 diffusion and inward air-convection. At the end of the flutter period, the spiracles open fully with the consequence of an abrupt intratracheal O_2 increase to atmospheric concentration and a bursting outflow of CO_2. By the cyclic discharge of the CO_2 water vapour loss is inevitable, but it is restricted mainly to this short burst phase. During vacuum increase in the tracheal system, the pupal abdomen passively shortens (Schneiderman and Schlechter, 1966; Hetz *et al.*, 1994; Fig. 3b). This shortening is a reaction of the compliant telescopable segments to the reduced tracheal volume (Gruber *et al.*, 1994). In spite of the abdomen giving way fully, the haemocoelic pressure becomes still more negative during shortening (see Fig. 5 in Slama, 1991). This clearly shows that no active muscle contraction is involved in this length-decrease. When spiracles open, the negative haemocoelic pressure forces the tracheae and the more rare air sacs to dilate and inspire atmospheric air. The mechanism for inspiration is driven by the antagonistic system of deflation of the partly specialized tracheae owing to O_2 consumption during closure of the spiracles and the subatmospheric haemocoelic pressure, which causes full inflation of the elastic tracheae as soon as the spiracles open and intratracheal pressure becomes atmospheric. The intratracheal vacuum cannot fall beyond the negative pressure of the haemocoel. Owing to the about 80% N_2 in the inspired atmospheric air, there is still a high gas volume in the tracheae after O_2 consumption, which should normally prevent a full collapsing of the tracheal system. It is therefore difficult to understand why, in living fleas, tracheae periodically collapse without pressure pulses (Herford, 1938). From the observations that saturniid pupal tracheae tend to collapse (Levy and Schneiderman, 1966c) and that in histological cross-sections of pupae of pierid butterflies, the diameter of distal tracheae looks slitlike, it may be concluded that the O_2-dependent volume reduction might not affect the tracheal system equally along its entire length but preferentially the distal blindly ending parts. In contrast, those tracheae which function as 'connection tubes' e.g. near the spiracles, at the bases of wings and legs, in the mouthparts and antennae (L. T. Wasserthal, in press) preserve a stable round cross-shape under these conditions. This would have the advantage that at the beginning of the flutter period, when the intratracheal pressure becomes atmospheric, the inspired air is efficiently sucked into these distal endings. In tracheae of specimens freeze-fixed under different conditions, the morphological changes are much less conspicuous and the structural correlate of the elasticity is visible only under the electron microscope. The elasticity of these distal tracheae is facilitated by an

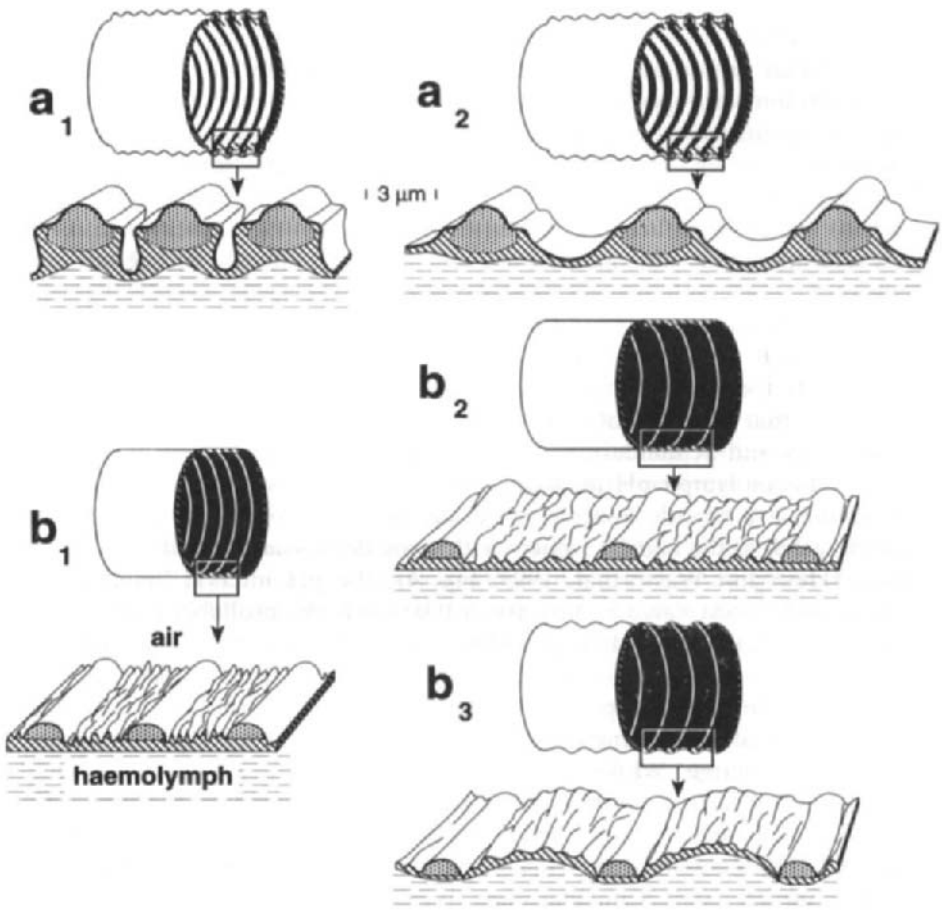

FIG. 5 (a–b) Adaptations of tracheae with 'simple' spiral taenidia to overatmospheric (a) and subatmospheric (b) haemocoelic pressures. (a) In the caterpillar (L5) of the hawkmoth *Panogena lingens*, the intima resists high haemocoelic pressure due to a massive keeled taenidium and a rigid intertaenidial membrane arced into the haemocoel (a_1). Even in the stretched and bent condition (a_2) the intertaenidial membrane bulges towards the haemocoel. (b) In the pupa of the butterfly *Pieris brassicae*, the (wing- and thoracic) tracheae have less pronounced, flat and irregularly arranged spiral taenidia with airsac-like folded intertaenidial membrane (b_1). This can be extended along the longitudinal axis of the trachea during growth and bending (b_2). When the intratracheal pressure becomes subatmospheric (during spiracular constriction) due to O_2 consumption, the intertaenidial membrane is bulged into the tracheal lumen, reducing the tracheal volume (b_3). Drawings are based on SEM and TEM photographs of specimens fixed under specified conditions.

airsac-like cuticle between the spirally arranged taenidia (Fig. 5b). While the variability of the cross-shape is limited by the spiral taenidium, the air sac-like intertaenidial intima can be distended outward or inward, thus enabling tracheal volume changes. The subalar gas-filled space in rhopaloceran pupae, which communicates with the tracheal system via the spiracles of the first abdominal segment, probably serves as a gas reservoir (Bourgogne, 1951). It may additionally play a role in the regulation of the internal pressure, because the inner surface of the wing sheath facing this gas reservoir consists of a very thin deformable membrane, which must be affected by haemocoelic and intratracheal volume and pressure changes.

A subatmospheric pressure and volume decrease of the tracheal system during closed phase can arise only if the intratracheal CO_2 does not compensate for the consumed volume of O_2. There are experimental data suggesting that the bulk of CO_2 (90%) leaves the haemolymph during the burst (Levy and Schneiderman, 1966a). This idea is supported by the fact that the haemolymph pH in papilionid pupae decreases during the closed phase and at a slightly lower rate during the flutter phase of the spiracles and increases to the highest value – with some delay – at the end of the open phase (Hetz and Wasserthal, 1993; Fig. 4); the pH interval between the highest and lowest value is only about 0.025 to 0.08, probably owing to an efficient haemolymph buffering system. In *Pieris brassicae* O_2-uptake has been shown to be associated with the end of the CO_2-burst, which does not fully match the model of passive suction ventilation (Crozier, 1979). In this species, there must be some (mechanical?) force, which separates O_2 uptake from CO_2 discharge. While the flutter is induced by the low $P\ O_2$ probably via the central nervous system, the CO_2 burst is induced by the high CO_2 in the haemolymph, possibly by (peripheral) receptors that are sensitive to some CO_2 accumulation dependent parameter (Levy and Schneiderman, 1966b; Burkett and Schneiderman, 1974). It is also known that the (denervated) closer muscle of the spiracle opens (relaxes) under high CO_2 concentration (Beckel and Schneiderman, 1957; Burkett and Schneiderman, 1974). The occurrence of spiracles with antagonistically operating closer and opener muscles, as in Hymenoptera and papilionid Lepidoptera (Schmitz and Wasserthal, 1996), suggests, however, that a direct influence of CO_2 upon muscle action is not probable.

The rather persistent negative haemocoelic pressure and the specialized elastic tracheae are prerequisites for suction ventilation. This mechanism seems to be the most economic system for survival during long periods. It relies on unavoidable O_2-consumption and the closing work of the tiny spiracular muscles. The tracheae hardly consume energy for deflation or dilation. Locally restricted supply, as may be possible in caterpillars, is avoided in pupae during transition from subatmospheric to atmospheric intratracheal pressure at the beginning of the flutter phase. The tracheal system can be supplied via a single spiracle, because the entire tracheal system dilates if the intratracheal pressure

becomes atmospheric. Unispiracular supply has been reported in *Manduca* pupae (Slama, 1988). In *Attacus atlas* and large papilionid (*Troides* and *Ornithoptera*) pupae, the quantity and velocity of CO_2-emission during a burst increases with number and surface of open spiracles and the average number of opened spiracles is 4–5 and 8–13 respectively (calculated from the CO_2-emission curves with and without experimentally sealed spiracles: Hetz and Wasserthal, in prep.). In pupae of *Galleria mellonella* inspiration is performed through the anterior two pairs of abdominal spiracles whereas CO_2 is released from the last abdominal spiracles, thus performing a unidirectional airstream during the flutter phase of suction ventilation (Kuusik *et al.*, 1996). If a pupal spiracle is sealed by detritus, the large number of spiracles guarantees gas exchange by some other spiracle(s). However, a destroyed permanently open single spiracle has the consequence of continuous diffusion with the disadvantage of higher loss of water vapour. With regard to O_2-supply, suction ventilation is a diffusion event combined with mechanical ventilation, without the involvement of pumping muscles and thus without pressure pulses. Positive pressure pulses by abdominal muscles, which may also have a ventilatory effect, are also produced by lepidopterous pupae (Slama, 1988, 1991; Kuusik *et al.*, 1996). It is, however, unclear which of the described types of active movement are obligatory in fully resting pupae, which are reactions to stress or disturbances and which represent forms of specific pupal activity. Although Slama (1988) also describes typical effects of suction ventilation in moth pupae, he argues that 'actual ventilation is brought about by genuine pulsations in haemocoelic pressure' of an unspecified all-comprising 'coelopulse system'. The effects of fluttering spiracles are ascribed to 'special extracardiac pulsations' (Slama and Coquillaud, 1992). However, since intratracheal pressure becomes more negative (!) when the abdomen shortens (!) and the abdomen elongates while the intratracheal pressure becomes atmospheric also during the microcycles (Fig. 3b), a pulsating mechanism other than the spiracular activity responsible for brief pressure changes during flutter phase is not detectable. There is evidence for active ventilatory movements at rest only in the above-mentioned coleopteran and hymenopteran pupae and in adult insects (see below).

4 Adaptations of circulation for eclosion and morphogenetics

4.1 HEARTBEAT ACTIVITY ADAPTED TO PREPARATION FOR EMERGENCE AND WING SPREADING

In the pharate adult very regular heartbeat reversals become established within the last day before eclosion in various insects (Gerould, 1933; Wasserthal, 1975b; Jones, 1977). Some hours before eclosion a stereotypic sequence of abdominal movement patterns is performed, which is very

conspicuous in saturniid pupae (Truman, 1972). Forward beating periods of the heart coincide with periodic lateral abdominal contractions, thus facilitating the loosening of the new cuticle and tracheae from the old ones within the abdomen as a preparation for shedding. Loosening of the tracheae in the anterior part of the body is probably helped by a transient local haemolymph reduction during backward beating periods of the heart. Two hours before eclosion in *Attacus* (2.5 h in the butterfly *Caligo* and a few minutes in the hawkmoth *Sphinx ligustri*) the heart begins to beat continuously forward with an accelerated pulse rate. The accumulation of haemolymph in the anterior part of the body increases the tension and facilitates the rupture of the pupal cuticle by violent shrugging of the wing bases. The adult leaves the pupa and cocoon by abdominal peristaltic contractions and crawling with the legs. In schizophore Diptera, the distending of the ptilinum for rupturing the puparium combined with general haemolymph pressure increase (Cottrell, 1962) represents a similar mechanism. In saturniids and butterflies which remain hanging at their cocoon or pupal exuvia, the wing spreading begins after some minutes or seconds respectively. Hawkmoths with terrestrial pupae often have to dig through the soil and find a suitable substrate, which can last from minutes to hours, before wing spreading begins (Truman and Endo, 1974). During this searching phase heartbeat reversals occur again. During wing spreading in all species observed, the heart beats forward with an accelerated pulse rate (Queinnec and Campan, 1972; Moreau, 1974; Wasserthal, 1975a,b; Tublitz and Truman, 1985). Most of the abdominal haemolymph is pumped, however, under the high pressure of the abdomen muscles directly through the waist into the thorax and the wing buds. This pressure at the beginning of wing spreading is so high that it often disturbs the action of the heart, resulting in quivering inefficient pulses. *Attacus*, with a ligated anterior aortal opening and a ligated head haemocoel, inflate their forewings fully with only little delay (Fig. 6a). The posterior margin of the hind wings, which is inflated at the end of wing spreading, however, spreads only if the heart is allowed to participate in pumping (Fig. 6b). A similar incomplete expansion occurs in individuals with a reduced haemolymph content in the pupa. The accelerated forward beating of the heart lasts 160 min in *Attacus*, 60 min in the butterfly *Caligo* and 48 min in the hawkmoth *Sphinx ligustri*. It thus exceeds the moment of reaching full wing length by 40 min in *Attacus*, 30 min in *Caligo* and 20 min in *Sphinx*. The continuous pulsating activity of the meso- and metatergal organs (POII and POIII) during wing inflation in *Bombyx* (Moreau and Lavenseau, 1975), *Attacus*, *Caligo* and *Sphinx* (L. T. Wasserthal, unpublished) may help to distribute the haemolymph within the lateral thoracic haemocoel along the wing hinge to provide an equal supply also to the posterior wing veins (Wasserthal, 1980). Disconnecting the wing veins from the POs during wing inflation by squeezing (clamping) the axillary cord has, however, no visible effect upon wing spreading in *Attacus*.

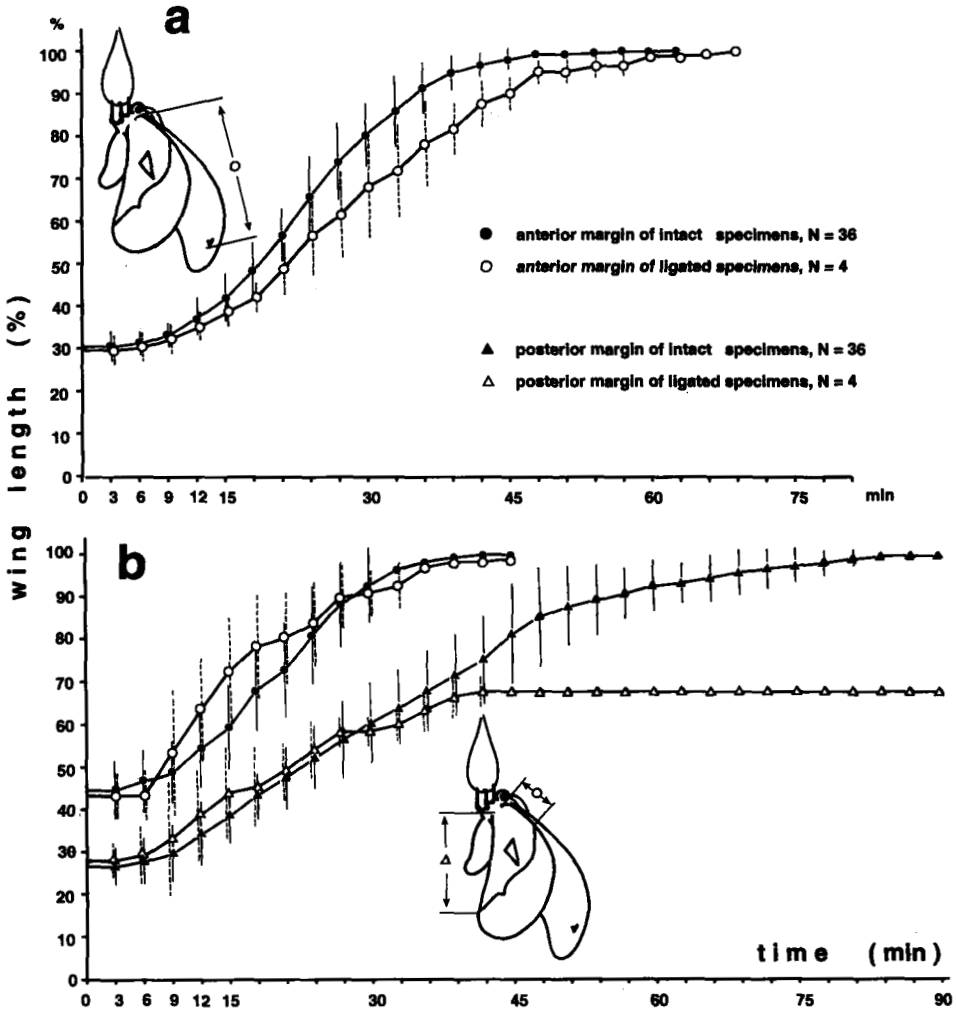

FIG. 6 (a–b) The (weak) effect of anterograde heartbeat for wing inflation in the giant silk moth *Attacus atlas*. (a) The inflation of the forewing is almost unaffected, if the heart pulse is prevented from leaving the anterior aortal opening by a ligature around the neck of the moth. (b) The posterior margin of the hindwing does not reach its full length after ligating the anterior aorta and haemocoel at the neck. 0 = initiation of wing inflation.

4.2 PROLONGED BACKWARD HEARTBEAT PERIODS AND THEIR INFLUENCE UPON DECREASE OF WING HAEMOCOEL AND INFLATION OF TRACHEAE AND AIR SACS

After wing spreading during cuticle hardening, haemolymph excess is sucked by the backward beating heart into the abdomen. During this time, the heart

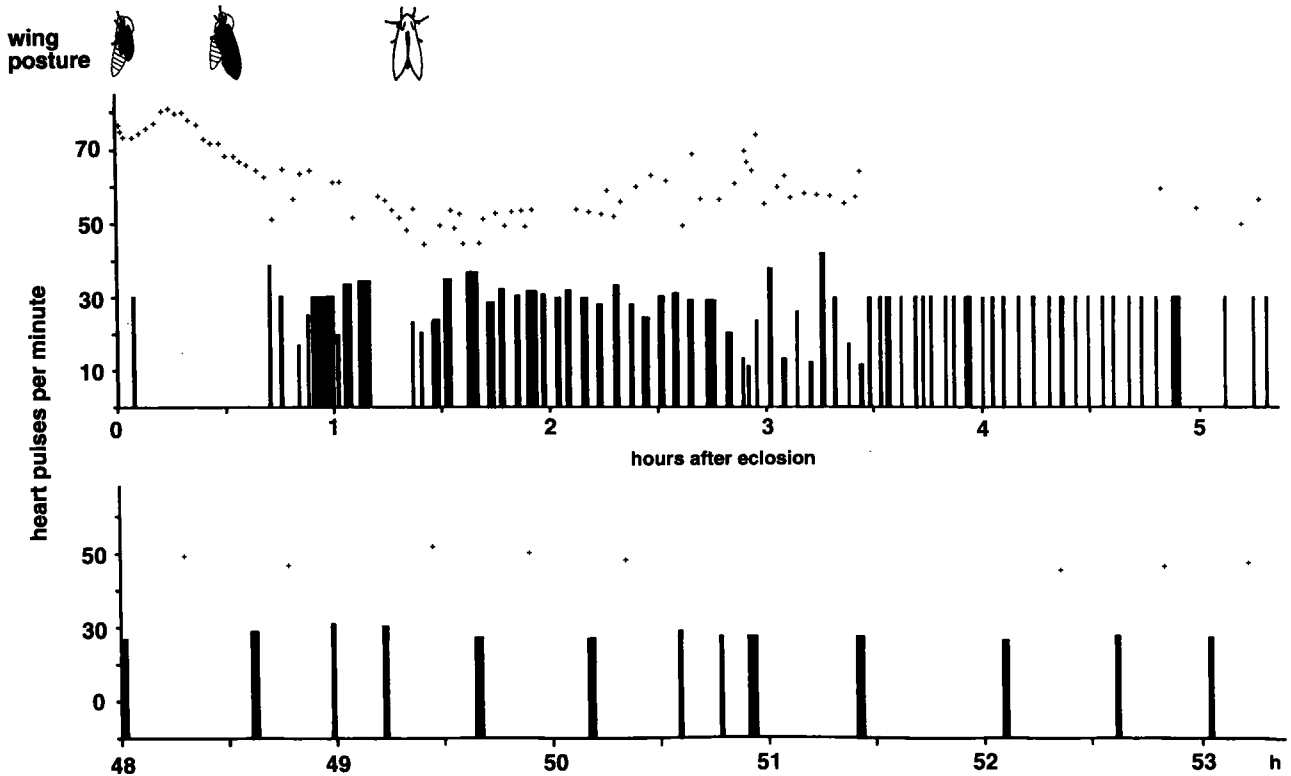

FIG. 7 Development of heartbeat frequency and relation between duration of forward- and backward pulse periods (black bars) in the hawkmoth *Sphinx ligustri* during and after wing inflation (female at T_a 23°C). The duration of the backward period is correlated with the haemolymph volume of the wings. This is highest just after wing inflation and stabilizes to a lower level 1 d after eclosion. Heartbeat reversal remains a regular event also in older hawkmoths, but due to the small haemolymph volume of the wings relative to that of the large body, the interval between backward pulse periods is rather great. Crosses = forward pulse rate, top of black bars = backward pulse rate.

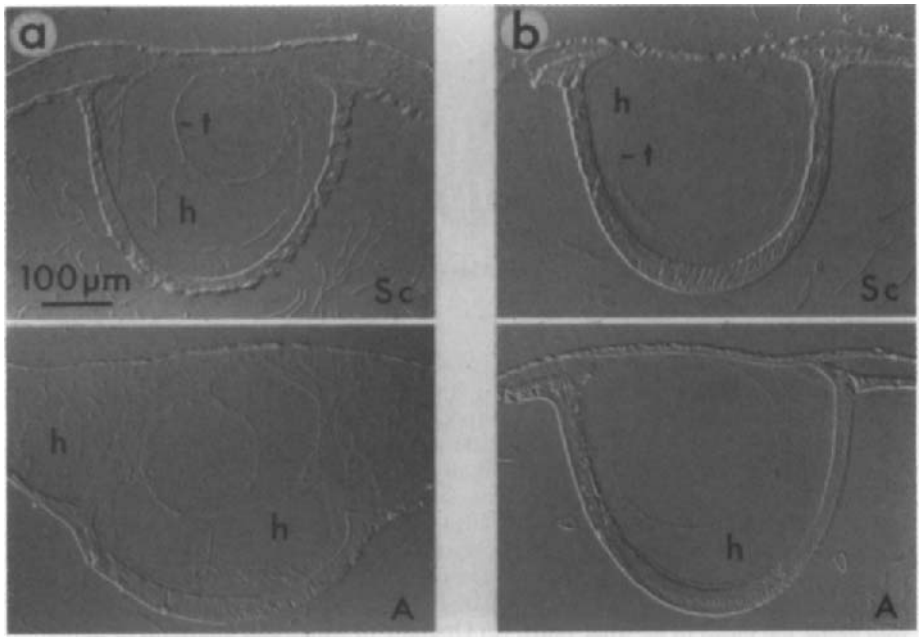

FIG. 8 (a–b) Cross-section of freeze-fixed veins from hindwings of the giant silk moth *A. atlas*. (a) Freeze-fixed just after wing inflation, 2 h after eclosion. Dorsal and ventral wing lamina are distant from one another. The hypodermal cells are cubic or stalked with haemolymph-filled spaces (h) between them. The lumen of the trachea (t) is rather small. The development of the posterior vein (A = Analis) is delayed relative to the anterior vein (Sc = Subcosta). (b) Freeze-fixed 24 h after eclosion. The wing laminae are tightly opposed and sclerotized with only flat hypodermal cells. Haemolymph (h) is reduced and restricted to the vein sinus around the distended trachea (t).

exhibits prolonged backward pulse periods combined with extended activity periods of the POs (Wasserthal, 1975a,b; Fig. 7). In the hawkmoth *Sphinx ligustri* the prolonged backward pulse periods last about 20 min. Then a second forward pulse period of about 20 min follows, during which the dorsal wing position changes into the lateral 'body hiding' position (Fig. 7). In *Attacus* the corresponding 'wing-opening' follows 20 min after onset of heartbeat reversals. There is a clear correlation between the original haemolymph content in the wings and the duration of prolonged backward beating of the heart. In the abdomen, the water content of haemolymph is reduced during postecdysial diuresis (Wigglesworth, 1963; Nicholson, 1976). The loss of water increases haemolymph viscosity and changes the haemocoelic pressure from positive to

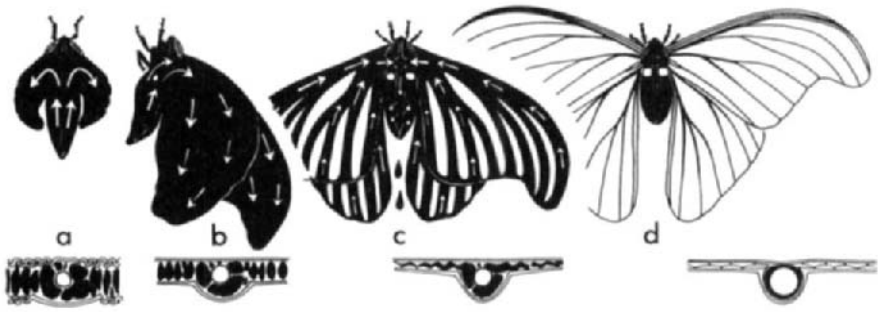

FIG. 9 The main circulatory events during wing inflation and sclerotization in saturniid silkmoths. The cross-sectioned wing-lamina and -vein in the lower figures show the changing relations of haemocoelic (black) and tracheal lumen (white) corresponding to the upper developmental states. Black with arrows = haemolymph with flow direction. (a) At the beginning of wing inflation: pumping of haemolymph through the haemocoel of the waist by contraction of abdominal intersegmental muscles into the anterior body and wing buds. (b) At the end of wing inflation. Abdominal contraction continues to press haemolymph into the wing buds. The anteriorly directed heartbeat is of importance only for inflation of the outermost part of the hindwing. The permanent pumping activity of the thoracic HPOs assures even haemolymph distribution along the bases of all wing veins. (c) Opening of wings and initiation of depletion of wing haemolymph excess by coordinated and intensified backward beating of the heart. The haemolymph now reappears in the abdomen, where it is dehydrated via excretion of water. (d) Twelve hours after eclosion, the haemolymph is reduced to about one-fifth of its original volume. The air sacs of the anterior body are inflated, partly occupying the haemocoelic space. The walls of the air sacs are under slight tension. The air sacs of the first abdominal segment are also widened and contribute to sealing the haemocoel of the anterior body compartment against that of the abdomen. The total volume of the abdomen is greatly reduced.

negative. This reduction of haemolymph volume causes, at first, upper and lower wing lamina to approach, so that only the wing veins contain haemolymph (Figs 8a, 9a–c); if there is no more compliance in the outer exoskeleton and the cuticle becomes sclerotized, the further haemolymph reduction induces the inflation of air sacs and of specialized tracheae, which during metamorphosis have been substituted for many of the simple tracheae with coiled intima (Fig. 8b, 9d). This process sets the tracheal walls under a basic elastic tension by the negative pressure, which from now on exists in the haemocoel. This basic negative pressure has been described to occur in adults of various insects, although it may transiently be increased by ventilatory or oviposition movements (Slama, 1976). In contrast to this process in pupae, the maintenance of this negative haemocoelic pressure is facilitated by the elastic force of the distended tracheae in relation to the low haemolymph content.

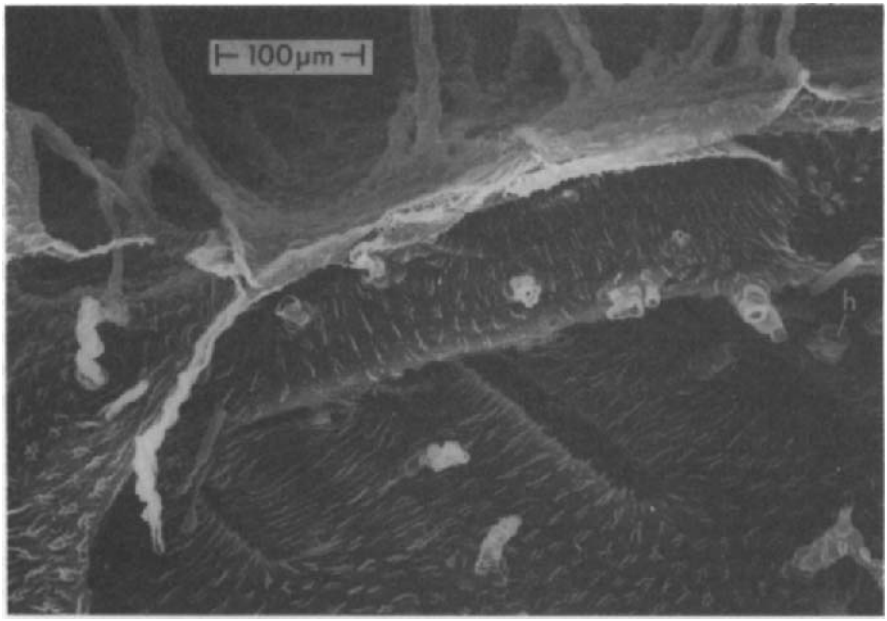

FIG. 10 View into the lumen of the expanded huge air sac in the head of a freeze-fixed death's head hawkmoth *Acherontia atropos*. The haemocoel is reduced to narrow tubes which traverse the tracheal 'aerocoel'.

5 Circulation and respiratory gas exchange in adults

5.1 DISPLACEMENT OF MOST OF THE HAEMOCOEL BY A SPACIOUS 'AEROCOEL' AND PARTITION OF THE HAEMOCOEL

In the head, wing veins and legs, the tracheal system occupies most of the interior like an 'aerocoel'. The large air sacs in the waist-region (first abdominal segment in higher Diptera, and metathorax and first abdominal segment in Lepidoptera) also become distended in fully developed adults (Wigglesworth, 1963; Faucheux, 1974). The spacious air sacs in the head and metathorax/first abdominal segment of Lepidoptera are traversed by narrow haemocoelic channels (Fig. 10). They are very numerous and seem to enlarge the contact between haemolymph and tracheal surface possibly for CO_2 transport. They have certainly a stabilizing and distending function for these air sacs. Some of them contain nerves (Brocher, 1920). The anterior abdominal air sacs serve, among other functions, as a barrier between the haemocoels of the anterior and posterior body, thus contributing to the establishment of a partitioned haemocoelic system. This compartmentation is morphologically manifested by the integration of the 1st abdominal segment as the propodeal

segment into the thoracic part and by the typical narrow waist between the 1st and 2nd abdominal segments in aculeate Hymenoptera. In addition, large air sacs occur in the anterior abdomen in bees, where an efficient thermal isolation has been described (Heinrich, 1976). In Lepidoptera, the septum additionally contains muscles (Hessel, 1969), connective tissue and constitutive fat body which becomes more densely arranged after the abdominal volume has been reduced by postecdysial diuresis. This fat body also persists during starvation. In scarabaeid beetles, the partition of anterior and posterior haemocoel is established in a different way (see below). In higher flies and beetles both haemocoel compartments are only connected by the dorsal vessel, since they lack a ventral diaphragm and the ventral nerve cord is concentrated in the anterior part of the body (Richards, 1963). In adult Lepidoptera and Hymenoptera there exists, however, a ventral passage through the perineural sinus (PNS).

5.2 HAEMOLYMPH OSCILLATION AND TRACHEAL VENTILATION AT REST

After establishment of the partition, at rest, the haemolymph oscillates between the anterior body and abdomen. In higher flies and beetles, periodic heartbeat reversal is alone responsible for the haemolymph oscillation (Figs 11, 13, 14, 15, 17). In higher flies and in scarabaeid beetles the aorta ends with its anterior opening in the posterior part of the head behind the brain (Normann, 1972; L. T. Wasserthal, unpublished). During backward transport haemolymph leaves the heart through a caudal unpaired opening (scarabaeid beetles) or paired openings (*Calliphora*) (Wasserthal, 1982b). In Lepidoptera and Hymenoptera the aortal opening ends in front of the brain and the heart has no specific caudal openings. Coordinated with the heartbeat periodicity a discontinuous haemolymph flow through the PNS supports the oscillation mechanism in Lepidoptera and the honey bee (see below).

5.2.1 *Diptera*

In higher (cyclorrhaphic) Diptera, such as *Calliphora*, with large air sacs in the anterior abdomen, the compartmentation is very conspicuous. Heartbeat reversals occur continually at rest, during grooming and even during feeding. The periods of heartbeat reversal are relatively short (Figs 11, 12). The forward pulse periods last 21 ± 12.6 s, and the backward pulse periods last 6.3 ± 4.85 s at 22°C ($N = 5, n = 7490$). The rate of the forward pulses is lower, $3.2\ s^{-1}$, than that of the backward pulses, with $4.0\ s^{-1}$ ($N = 6, n = 987$) (L. T. Wasserthal, unpublished). This corresponds to the observations of Gerould (1933), who counted the heart pulses in the crane fly *Pachyrhina ferruginea*, a representative of the other dipteran suborder Nematocera, but it is in contrast to interpretations that in *Calliphora* and *Phormia regina* the shorter periods with the higher pulse rate are forward pulse periods (Queinnec and Campan, 1975;

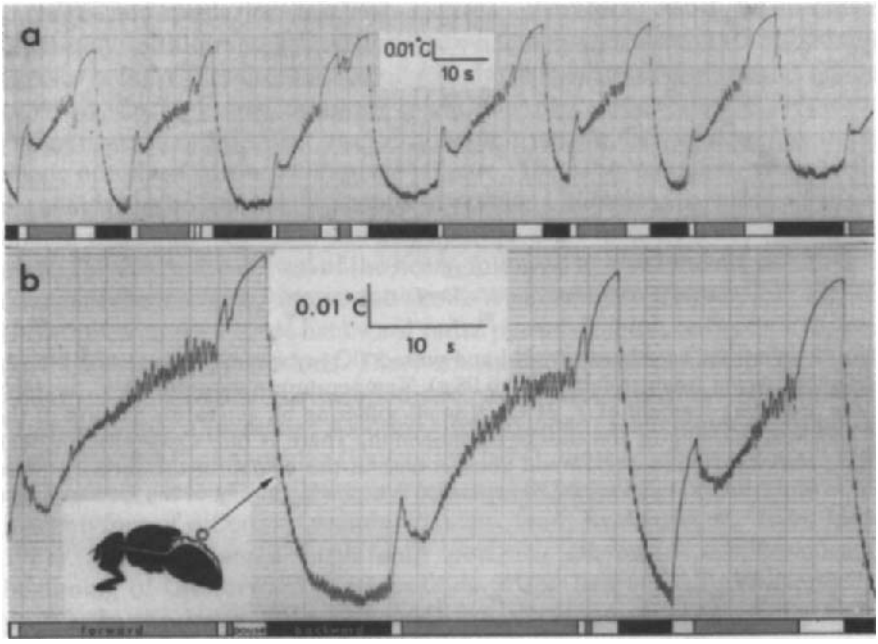

FIG. 11 (a–b) Periodic changes in thermal conductivity of the abdomen in *Calliphora*, recorded by a thermistor site heated to an excess temperature of 1.8°C (T_a = 22°C). Conductive cooling is increased during backward pulse periods, suggesting a higher density by increase of abdominal haemolymph content at the cost of tracheal air volume. The spikes represent the convective cooling effect of the single heart pulses (from Wasserthal, 1982b). (b) = detail from (a).

Thon, 1982; Angioy and Pietra, 1995). An exact determination of heartbeat frequency in flies is difficult, because, both, forward and backward pulse rates are highest at the beginning and lowest at the end of each period, when in most individuals pulse pauses of up to several seconds occur (Fig. 11). This decrease in pulse rate suggests an increase of flow resistance at the end of each period. During backward beating, the haemolymph enters the greater ostia of the first and second heart segments. This anterior part of the heart belongs functionally to the anterior body haemocoel by its position anterior to the large abdominal air sacs. The valve flaps of only these ostia are oriented towards the caudal end. Haemolymph is sucked from the lateral thorax coming from the wing and haltere muscles and from the paired lateral accessory pulsatile organs, which aspire wing haemolymph. Haemolymph, leaving the heart through the posterior openings, accumulates in the abdomen. As this has only a slightly compliant integument and only minute changes of its outer shape could be measured (L. T. Wasserthal, in preparation), the increased haemolymph volume must be compensated by the decreasing volume of the large air sacs

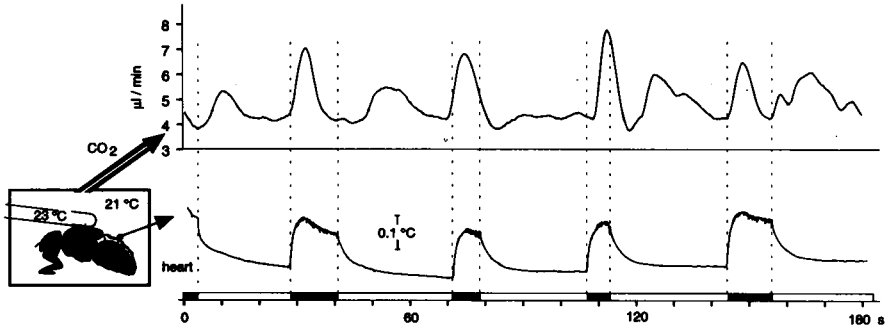

FIG. 12 Periodic heartbeat reversal and pulsed CO_2-emission in the resting blowfly *Calliphora vicina* (mature female of 0.08 g). Temperature measurement at the level of the 3rd heart segment at T_a 21°C. A small soldering bit serves for fixation of the fly and heat-marking of the thorax haemolymph. There is one CO_2-emission peak during backward pulse period and another one in the course of the forward pulse period of the heart. The lowest CO_2-emission is at the beginning of the forward pulse period.

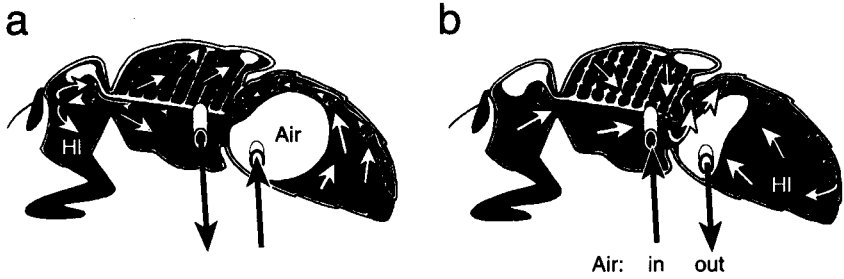

FIG. 13(a–b) Compensatory changes in the tracheal volume, caused by haemolymph oscillation between anterior and posterior body in *Calliphora vicina*. (a) During forward pulse period of the heart, haemolymph (Hl) leaves the aorta in the head. The tracheal volume in the anterior body decreases. (b) During backward pulse periods, haemolymph enters the anterior heart ostia in front of the pair of large abdominal air sacs and leaves the caudal heart through a pair of excurrent ostia. The increasing haemolymph volume partly displaces the volume of the air sacs (from Wasserthal, 1982b, modified). Expiration/inspiration of anterior and posterior body alternate due to periodic haemolymph shift, because the tracheal systems are separate.

(Fig. 13). The augmentation of haemolymph content in the abdomen at the cost of the air volume can indirectly be deduced from the higher thermal conduction measured with heated thermistors during backward beating of the heart (Fig. 11). The longitudinal connection of the abdominal air sacs with the thoracic tracheal system is very narrow or interrupted (Faucheux, 1974). The

anterior and posterior tracheal systems, therefore, must be ventilated separately and alternately by the periodic haemolymph shift. As the abdominal air sacs in these flies are said to collapse during maturation of ovaries (Evans, 1935), it was suggested that they might play no essential part in respiration (Wigglesworth, 1963). But even in pregnant female *Calliphora*, they occupy about one-third of the abdominal volume. The CO_2-emission, measured in parallel with the heartbeat periods in resting *Calliphora*, is continuous with one maximum during backward pulse period and a minimum at the beginning of the forward pulse period of the heart, followed by a second maximum in the course of the forward pulse period (L. T. Wasserthal, in preparation; Fig. 12). Blowflies with very short backward pulse periods exhibit only one CO_2-peak during forward pulse periods. There are similarly large air sacs in the anterior abdomen in Tipulidae, Anisopodidae, Sciaridae, Culicidae, Psychodidae, Stratiomyidae, Tabanidae, Asilidae, Bombyliidae, Rhagionidae, Syrphidae, Muscidae and Calliphoridae (Faucheux, 1974), most of which are good fliers.

The occurrence of ventilatory movements has previously been reported from inactive flies of different families (Plateau, 1884; Krafsur *et al.*, 1970; Miller, 1981). In *Calliphora* a single slow pumping movement accompanies the beginning of the forward pulse periods of the heart (L. T. Wasserthal, in prep.). In the large *Pantophthalmus tabaninus* conspicuous oscillations of thoracic temperature are synchronized with discontinuous increase of O_2-consumption (Bartholomew and Lighton, 1986). These cycles are rather slow and probably reflect a different mechanism of cyclic variation in metabolic activity.

5.2.2 *Coleoptera*

In Coleoptera only a few observations are available, containing data about circulation. On the basis of visual observations, heartbeat reversal has been described to occur as a regular event in *Prionus laticollis*/Polyphaga (Gerould, 1933). In *Aptynus displosor*/Adephaga (Dubuisson, 1930), and in recent electrophysiological studies, heartbeat reversal is thought to occur more exceptionally, '. . . but circulatory problems are unlikely to be brought about given the open vascular system that is common in insects' (Ebara *et al.*, 1990). Very regular heartbeat reversal has, however, been recorded with microthermistors in intact, resting representatives of both coleopteran suborders and is certainly a normal event of circulation (Wasserthal, 1982b and Figs 14, 15). In the large cetoniine beetle *Goliathus goliathus* the backward pulse periods last 0.7 ± 0.08 min ($n = 126$ at T_a 21°C) with a pulse rate of 80–90 per min whereas the longer forward pulse periods have a duration of 1.1 ± 0.14 min, $n = 122$ with a lower pulse rate of 75–80 pulses per min. In the dynastiine rhinoceros beetle *Oryctes nasicornis* the relations of the heartbeat periods are similar to those of the goliath beetle, but the forward pulse frequency can be slightly lower, identical, or more rarely, somewhat higher than that of the backward

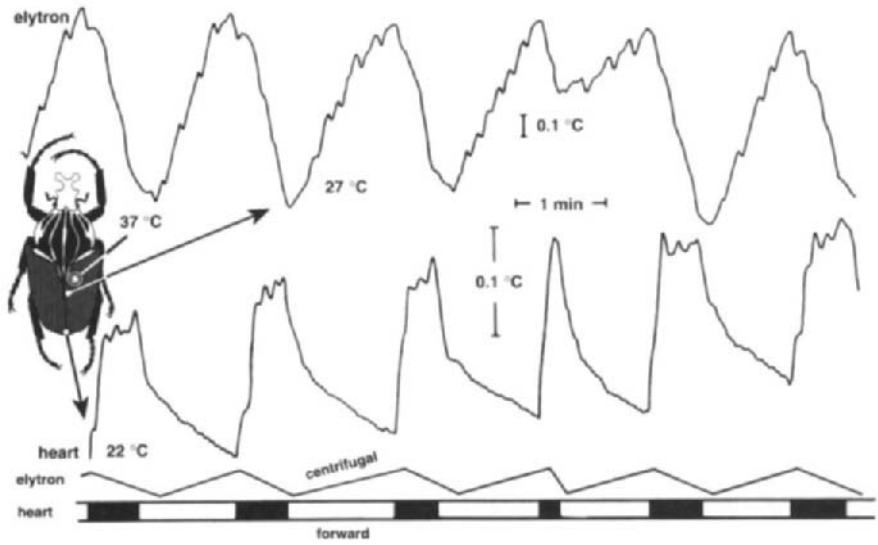

FIG. 14 Periodic heartbeat reversals and coordinated haemolymph flow reversal in the elytra of the scarabaeid beetle *Goliathus goliathus*. During backward beating, thoracic haemolymph – heated by a resistor to 37°C – arrives at the posterior heart (thermistor at the pygidium) indicating backward pulse period (black bar), while the elytral thermistor records a cooling effect, indicating the flow of unmarked distal haemolymph towards the wing base. During forward heartbeat periods, heat-marked haemolymph from the thorax and wing base flows distally.

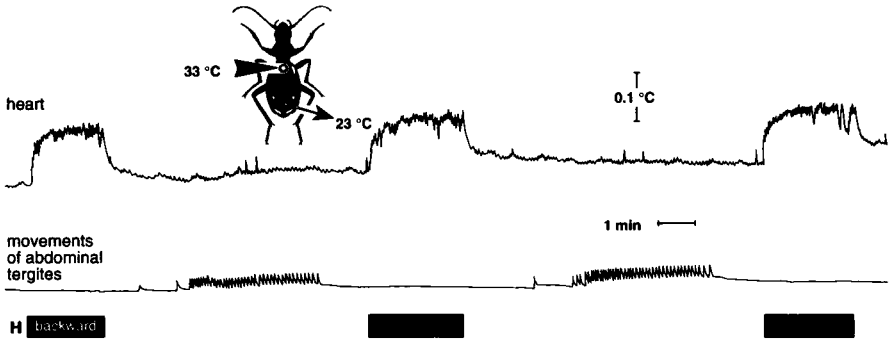

FIG. 15 Coordination of heartbeat periodicity (thermography with heat-marked thoracic haemolymph) and ventilatory movements of abdominal tergites (infrared-lightbeam-photocell-recording) in the desert carabid beetle *Thermophilum hexmaculatum*, T_a 23°C. The ventilatory bursts coincide with forward pulse periods of the heart.

pulses, depending on the individual. In general, the data correspond to the frequency counts of Gerould (1933) in cerambycine beetles, who also found that the backward pulse frequency was slightly higher than that of the forward pulses. In the desert carabid *Thermophilum hexmaculatum* the backward pulse periods are shorter (2.0 ± 0.62 min, $n = 505$ at T_a 22°C) but have a much higher pulse rate with 60 ± 16.3 pulses per min than the forward pulse periods (7.76 ± 2.15 min, $n = 505$) with 30 ± 3.18 pulses per min ($N = 8$) (L. T. Wasserthal, unpublished; Fig. 15). As in Diptera the relatively higher backward pulse frequency in beetles may be a consequence of the posterior outflow opening of the heart. In beetles this is even larger than the aortal opening in the head, and discharges the haemolymph into a wide pygidial cavity (Wasserthal, 1982b).

The narrow passage between thorax and abdomen in the scarabaeid beetles seems to be largely sealed by the intestine and surrounding fat body, thus reducing the free space for – at least a rapid – haemolymph exchange. While the anterior part of the body with its appendages is extremely uncompliant, the abdominal volume can change due to its flexible tergites below the overlying elytra. The periodic shift of haemolymph between the anterior and posterior part of the body is compensated by the tracheal volume of the air sacs in both body compartments. In the transparent elytra of *Oryctes* a periodic volume change coordinated with heartbeat reversals could be visualized by time lapse cinematography and micrography (L. T. Wasserthal, unpublished; Figs 16, 17). In the goliath beetle, the periodic haemolymph inflow into the elytra during forward pulses and outflow during backward pulses has been recorded with thermistors during heat-marking of the basal elytron and anterior abdomen with a resistor (Fig. 14). In spite of the compliance of the abdomen, the increased haemolymph volume reduces the volume also of the abdominal air sacs. This can be deduced indirectly from the better thermal conduction (cooling of a heated abdominal thermistor site) during backward pulse periods.

In beetles such as *Oryctes* and *Melolontha*, abdominal ventilation movements are well known (Rathke, 1862). Abdominal air sacs are compressed by active depression of the tergites, which are kept in an inspiratory position by elastic resilin ribs (Andersen and Weis-Fogh, 1964). Metathoracic pumping as observed in swimming beetles *Hydrophilus* and *Dytiscus* (Brocher, 1931) and prothoracic pumping, accompanying abdominal ventilation movements, as described in prionine beetles (Miller, 1971) are a more exceptional situation. A number of studies dealing with respiratory gas exchange, describe discontinuous occurrence of respiratory phenomena. Discontinuous CO_2-discharge and O_2-uptake have been recorded in *Carabus* (Punt *et al.*, 1957) and in different tenebrionid beetles at rest (Bartholomew *et al.*, 1985; Lighton, 1985, 1988b, 1991). Oscillations of endogenous thoracic temperature in the range of a few minutes have been described in large Scarabaeidae and Cerambycidae, where they occur simultaneously with intermittent ventilatory activity (Bar-

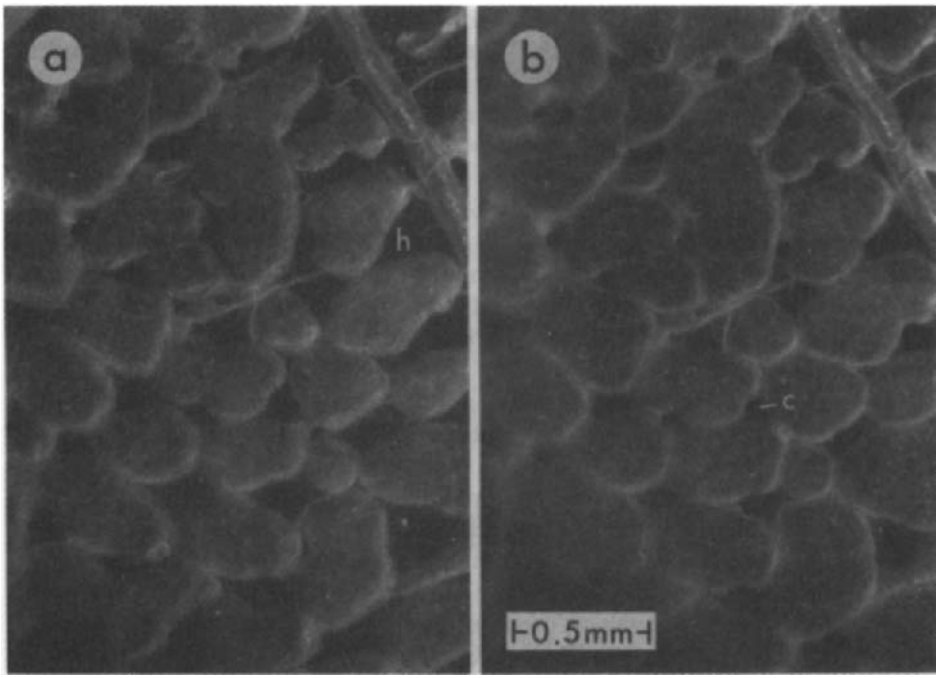

FIG. 16 (a–b) Photomicrographs of the interior of the transparent elytron of a living rhinoceros beetle *Oryctes nasicornis*, sleeping in a fixed position under the microscope. A reflex-free and sharp image could be achieved by gluing a cover glass on the intact cuticle. (a) Photograph was taken during forward pulse period of the heart. Air sacs are less distended and haemolymph-filled spaces (h) around the columns (c) are obvious. (b) Photograph was taken at the end of backward pulse period. The air sacs have displaced most of the haemocoel.

tholomew and Casey, 1977) or with discontinuous oxygen-consumption (Morgan and Bartholomew, 1982). In *Bruchus* cyclic CO_2-emission is associated with 'extracardiac pulses' (Coquillaud *et al.*, 1990), probably abdominal ventilatory movements.

In hibernating *Oryctes* under high humidity, CO_2-emission, recorded in parallel with heartbeat periods, is cyclic too, but the interburst phases with no measurable CO_2-output are generally much longer and not coordinated with heartbeat reversals. Heartbeat reversals do not lead to visible fluctuations in CO_2 emission, which is clearly correlated with depression bouts of the abdominal tergites (L. T . Wasserthal and T. Fincke, unpublished). It must, therefore, be concluded that CO_2 in these beetles is stored in the haemolymph during the interburst phase throughout several heartbeat periods. In addition, the recorded volume changes of the air sacs owing to the periodic haemolymph shift between the anterior and posterior body haemocoel might either serve for

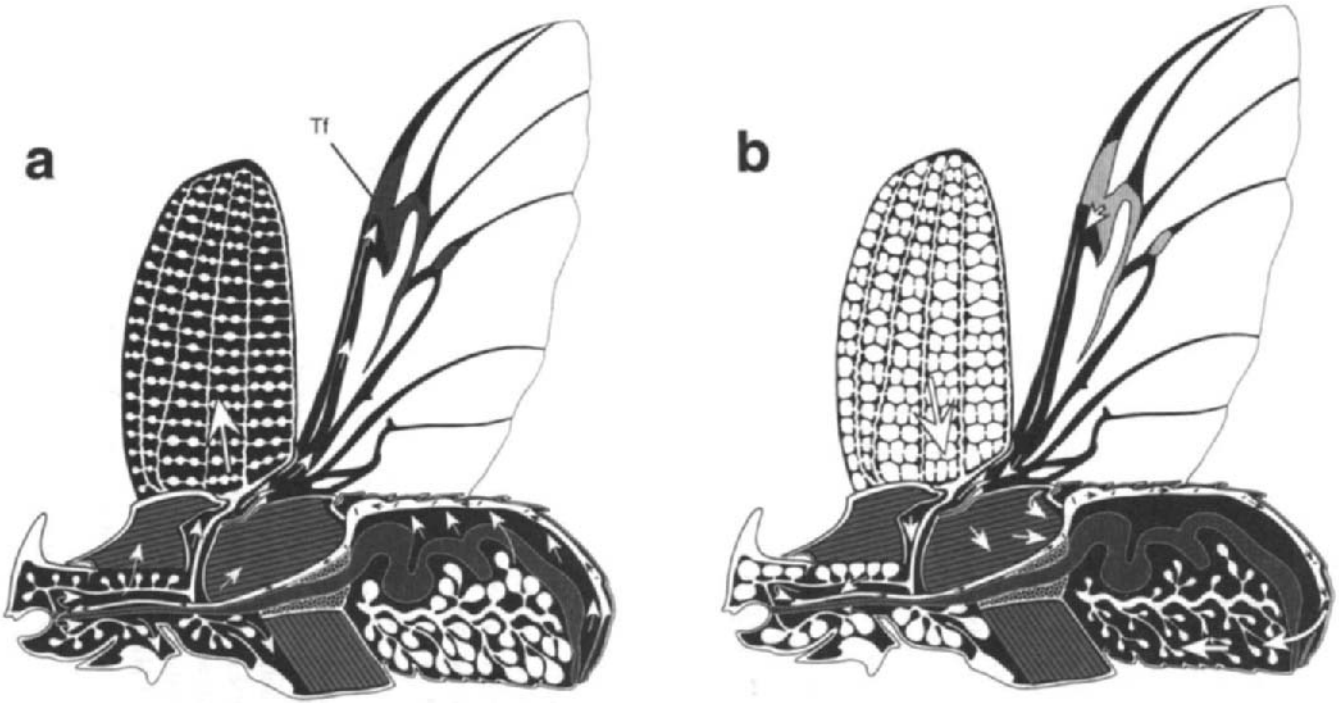

FIG. 17 (a–b) Relationship between haemolymph volume and tracheal volume in the rhinoceros beetle *Oryctes nasicornis*. (a) Haemolymph accumulation and reduction of tracheal volume in the anterior body with elytra during forward beating of the heart. (b) Reduction of haemolymph volume and increase of tracheal volume in the anterior body during backward beating of the heart. Modified from Wasserthal (1982b). As the hindwing contains no air sacs, haemolymph volume is possibly reduced under inward bending of the elastic veinal cuticle (stippled area) at the transverse wing fold (Tf). The tension of this cuticle may suck haemolymph distally like a pipette during forward beating of the heart.

the inspiration of fresh air alternately in the anterior and the posterior parts of the body, assuming the spiracles are open, or, if the spiracles are closed during interburst phase, a mixing of the air within the tracheal system of the entire body might result. As a compensatory volume change, air might partly be exchanged between the anterior and posterior parts of the body along the longitudinal trunks described by Straus-Durckheim (1828). In spite of these longitudinal trunks of the tracheal system, the recorded abdominal pumping movements do not measurably affect the volume of the anterior air sacs in scarabaeid species owing to their uncompliant exoskeleton in the anterior part of the body. Earlier experiments, which led to the conclusion that the thoracic air sacs inflate during abdominal ventilatory movements, were done in specimens with cut wings or other perforations of the anterior body (Rathke, 1862). A similar model of an alternation of thoracic and abdominal expiration during abdominal ventilation was postulated by Graber (1877). In contrast to the rhinoceros beetle, in the desert carabid, *Thermophilum hexmaculatum*, the discontinuous ventilatory movements are coordinated with heartbeat reversals and occur always during forward pulse periods (Fig. 15).

5.2.3 *Hymenoptera*

In Hymenoptera, heartbeat reversal has only been described after visual observations in a decapitated and wingless *Sphex*, Sphecidae and a moribund *Opheltes*, Ichneumonidae (Gerould, 1933). No recordings have confirmed the existence of heartbeat reversal in this insect order. The ventral diaphragm is suggested to play an important role in backward haemolymph transport (Freudenstein, 1928). In fact, haemolymph oscillation in the honey bee is performed by discontinuous activity of the heart and discontinuous transport through the PNS (Fig. 18). In the resting honey bee, impedance measurements showed intermittent bouts of high amplitude pulses of 3–6 Hz alternating with interburst periods with no pulses or low amplitude pulses with lower frequency of 1–3 Hz (Schwab *et al.*, 1991). Contact thermographs, measuring the actual temperature at the abdominal heart and the perineural sinus of sleeping honey bees with heat-marked thoracic haemolymph, revealed that long heart pauses coincide with the periodic backflow of haemolymph through the PNS (L. T. Wasserthal and W. Molsbach, unpublished; Fig. 18). While the prolonged periods of heart activity start a few seconds after onset of the abdominal ventilatory bout, the flow through the PNS is lowest shortly after the end of the intermittent abdominal ventilatory movements. The heart beats only forward! It is remarkable that the pulse frequency of the larger abdominal wall muscle system is higher (4 Hz) than that of the heart (2.16 Hz, both at 23°C). The abdominal movements are the motor for cyclic CO_2-discharge also in the adult honey bee (Fig. 18b). In the above-mentioned impedance measurements (Schwab *et al.*, 1991), the different types of pulses possibly represent the mixed effects of heart and abdominal wall muscles. The backward flow of

haemolymph through the PNS during heartbeat pause has the consequence of periodic accumulation of the haemolymph in the abdomen just at the moment when maximal hydraulic function of the haemolymph is needed for effective ventilation of the abdominal air sacs. Intermittent backflow of haemolymph has also been recorded in a cooling bumblebee (Heinrich, 1976), in which heart, abdomen wall and ventral diaphragm have been suggested to beat in a coordinated fashion with the same frequency when the insect is active. Discontinuous ventilation has been described in honey bees recently, however, with much longer intervals (Rothe and Nachtigall, 1989) or only under lower ambient temperatures between 12 and 15°C (Lighton and Lovegrove, 1990). Cyclic CO_2-emission seems to be widespread in resting Hymenoptera, such as *Bombus*, *Ammophila*, *Polistes* and *Urocerus* (L. T. Wasserthal and W. Molsbach, unpublished) and is performed in the formicine ants, *Camponotus* (Lighton, 1988a) and *Cataglyphis* (Lighton *et al.*, 1993). The relatively short intervals of the CO_2-bursts, about 0.5–2 min^{-1} at 25°C in the above-mentioned larger Hymenoptera, but also in *Camponotus*, are reminiscent of the duration of haemolymph oscillation periods in the honey bee and could likewise be coupled with a similar haemolymph oscillation mechanism. In ants, in which no ventilatory movements have been detected as yet, a suction ventilation mechanism regulated by spiracular activity, similar to that of lepidopterous pupae, has been proposed (Lighton, 1988a; Lighton *et al.*, 1993). In *Cataglyphis*, CO_2-bursts occur in longer intervals of about 1 per 20 min at 25°C simultaneously from the thoracic and the gastric spiracles! As during closure and flutter phase positive intratracheal pressure sometimes transiently arises, some additional factor, or active system, may be involved in this respiration mechanism (Lighton *et al.*, 1993). A simultaneous CO_2-emission of the anterior and posterior parts of the body could be performed under a slow abdominal shortening combined with an air and/or a haemolymph shift (i.e. a forward beating heart without backflow through the PNS from abdomen to the thorax). In *Apis* at the onset of the ventilatory burst, the abdomen contracts and in spite of the waist, the ventilation effect is perceivable as an increased (convective + conductive + evaporative) cooling effect also in the (heated) dorsal mesothorax (L. T. Wasserthal and W. Molsbach, unpublished). The propodeal spiracle, which is closed during abdominal expansion and which is open during abdominal contraction, is suggested to serve for expiration (Bailey, 1954). The rapid drop in intratracheal pressure during constriction in *Cataglyphis* and its remaining at a subatmospheric level also during flutter phase in contrast to the almost atmospheric pressure in the flutter phase of *Attacus* pupae, suggest that the negative pressure in the thoracic tracheal system might originate from some additional force. Assuming that, as in the honey bee, haemolymph is sucked by slow abdominal length- (volume-) increase and/or flow through the PNS while the heart stops beating after each ventilatory burst, an increased pressure drop in the tracheal system of the thorax or of the entire tracheal system could arise.

5.2.4 *Lepidoptera*

Heart activity in resting Lepidoptera is always discontinuous. The duration and frequency of heartbeat reversals are correlated with the haemolymph content in the wings. In recently eclosed individuals, backward pulse periods are longer and follow upon each forward pulse period (Wasserthal, 1975a,b; Fig. 7), whereas in fully developed adults with distended spacious wing tracheae, heartbeat reversals become shorter. In large-winged Saturniidae the duration of the backward pulse periods remains almost as long as the forward pulse periods (Fig. 19a). In hawkmoths and Noctuidae with relatively small wings and in butterflies with relatively large wings but extremely reduced vein haemocoel owing to extremely distended tracheae, the backward pulse periods appear at greater intervals. Therefore, backward pulses have been observed more rarely and only under certain conditions (Heinrich and Bartholomew, 1971; Heinrich, 1971) and have been interpreted as 'short and irregular' events (Queinnec and Campan, 1972). The heartbeat pause, which is normally performed at the transition from forward to backward beating, becomes a more frequent event in hawkmoths, noctuids and butterflies, interrupting the prolonged forward pulse periods several times. Forward pulse frequency in Lepidoptera is always higher than backward pulse frequency (Gerould, 1929a) and shows specific frequency fluctuations according to the abdominal length changes (Wasserthal, 1980, 1981). Periodic expanding of the abdomen is

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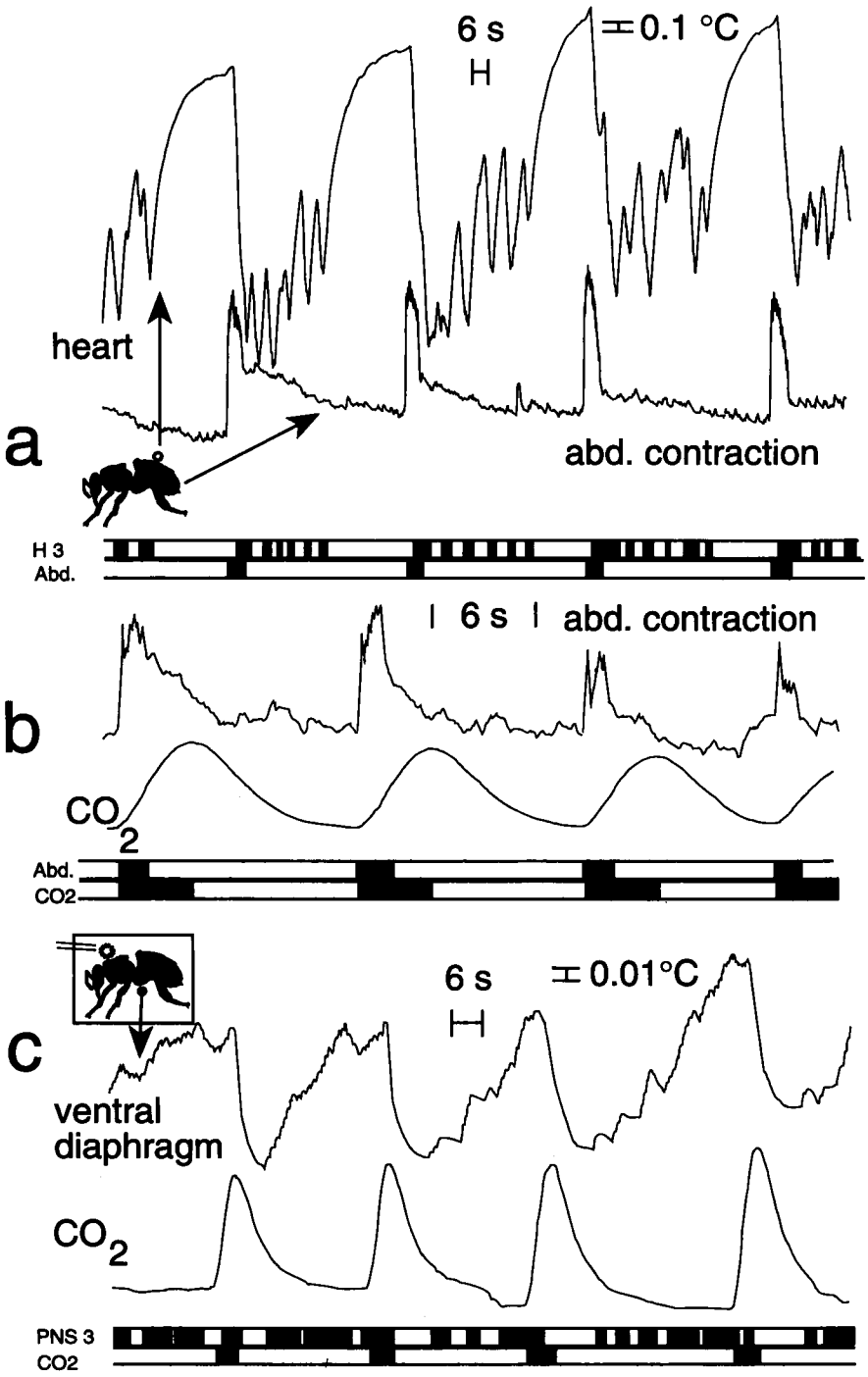
FIG. 18 (a–c) Coincidence of discontinuous ventilation and periodic haemolymph shift between anterior and posterior body by discontinuous (forward) pulse activity of the heart and discontinuous (backward) flow through the perineural sinus (PNS) in sleeping *Apis mellifera* at 25°C in full darkness (L. T. Wasserthal and W. Molsbach, unpublished). (a) Simultaneous measurement of heartbeat activity with a heated thermistor on the 3rd abdominal tergite and of abdominal length changes, recorded by an infrared-lightbeam photocell. The convective effect of each series of heart pulses leads to a steep cooling of the heated thermistor site (black bar in the signature). Single pulses are not resolved in the figure. Heartbeat pauses lead to a rewarming of the thermistor site (white bars). The bouts of abdominal contractions (black bar) coincide with the moment of transition from the long heartbeat pauses to prolonged beating of the heart which is always forward. The convective cooling effect of the heart pulses may be superimposed by conductive cooling (higher density owing to reduced air sac volume) and by evaporative cooling (owing to expiration). (b) Discontinuous ventilatory movements accompanied by CO_2 discharge. (c) Simultaneous measurement of discontinuous CO_2 discharge (URAS) and periodic haemolymph flow in the perineural sinus measured with a non-heated thermistor stuck onto the 3rd abdominal sternite while the thoracic haemolymph is heat-marked ($\Delta T = 2°C$). After the end of the ventilatory bout, the haemolymph flow through the perineural sinus is reduced (cooling phase = white bar); as this conspicuous cooling phase occurs after the steep one of the heart-curve, it cannot be effected by the same mechanisms. Most flow is during the long heart pause (heating phase = black bars). Single pulses of the ventral diaphragm are visible.


6 s
H
± 0.1 °C
heart
a
abd. contraction
H 3
Abd.
6 s
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b
CO2
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c
6 s
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ventral
diaphragm
CO2
PNS 3
CO2

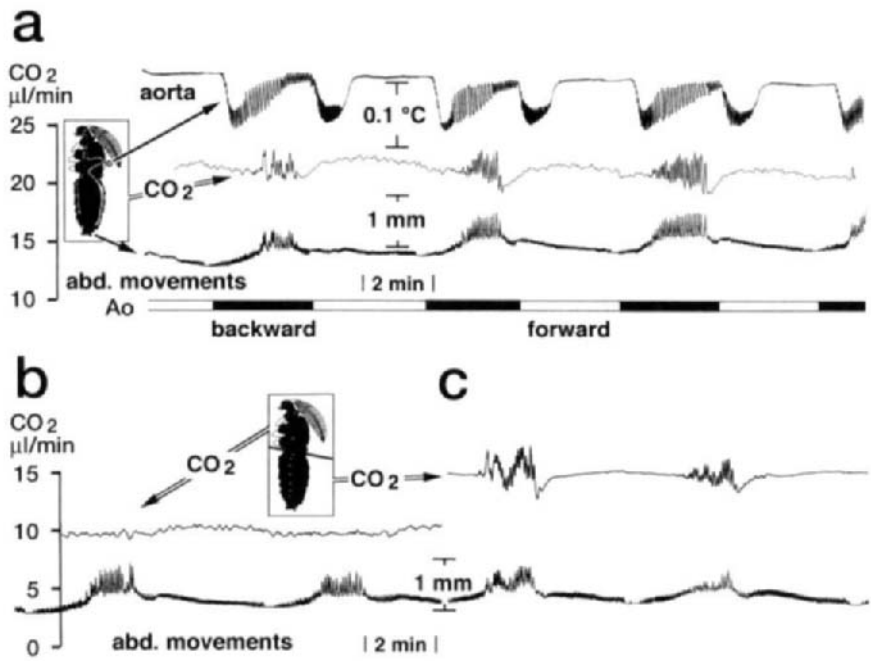

FIG. 19 Coordination of heartbeat reversal with discontinuous abdominal ventilatory movements and CO_2-emission in an adult resting *Attacus atlas* (2-day-old female at 25°C). The aortal pulses were recorded by a thermistor heated to 1.8°C above ambient. CO_2 output of the entire moth was measured via URAS. The ventilatory movements were monitored by a Hall sensor arranged about 4 cm laterally beside the abdomen (L. T. Wasserthal and T. Fincke, unpublished). (a) The ventilatory bouts coincide with the second half of the backward period of the heart. (b) The separately measured CO_2 output of thorax is continuous and not affected by the ventilatory movements. (c) The separately measured CO_2 output of the abdomen is also continuous but almost twice as high as that of the thorax and shows pronounced effects of the abdominal compression movements.

correlated with a decrease of forward pulse rate and abdominal shortening with its increase. In free resting intact Lepidoptera backward beating is accompanied by – often inconspicuous – slow abdominal length increases. In moths with unpaired thoracic accessory pulsatile organs (POs), the highest backward frequency coincides with the moment of pulse activity of the POs, mostly within the first third of the backward pulse period, that is, when most wing haemolymph is being transported (Fig. 20 and see below). In noctuids and hawkmoths, forward frequencies may change from pause to pause and periods with low forward pulse rate (Queinnec and Campan, 1972; L. T. Wasserthal, unpublished) may easily be misinterpreted as backward pulses. Pulse direction in these moths can be determined only if the metachrony of the contraction wave is analysed or some marker is used. Backward pulse periods occur more

regularly and coordination of heartbeat periodicity is more precise in vital individuals, capable of persistent flying. Also, heartbeat pauses in all intact free resting Lepidoptera examined as yet, occur only during abdominal length increase. It is assumed that abdominal lengthening renders the internal abdominal haemocoelic pressure more negative. It could be shown that during abdominal length increase, especially during heartbeat pause, haemolymph is forcibly sucked through the perineural sinus (PNS) (Wasserthal, 1980, 1981; Fig. 20). The ventral diaphragm, with its lateral undulations, continuously drives the haemolymph through the abdominal PNS caudally. It seems to serve mainly for haemolymph distribution within the abdomen. As the backward haemolymph flow through the PNS begins during the second half of the forward pulse period in *Attacus*, a haemolymph circulation occurs until the end of the forward pulse period and an equilibrium of both haemolycoelic and tracheal volumes is maintained within the anterior and posterior compartments. During the following heartbeat pause, the intensification of (backward) flow through the PNS leads to an increase of haemolymph volume in the abdomen and to a corresponding decrease in the anterior compartment. Whereas in the thorax this haemolymph deficit is measurable as a decline of thermal conductivity (increase of temperature with heated thermistors, owing to expanding air sacs with better isolation characteristics), the abdomen expands its total volume. The volume of the abdominal tracheal system therefore remains relatively constant on a maximal distended state during that phase.

5.3 BACKWARD HAEMOLYMPH TRANSPORT WITH BIDIRECTIONAL HEART OSTIA IN LARGE MOTHS, A SOPHISTICATED HIERARCHICAL SYSTEM OF 3 'VACUUM PUMPS IN SERIES'

In higher flies and beetles, the backward pumped haemolymph leaves the heart through the specific posterior openings, mentioned above. A transition of haemolymph from the heart through the ostia into the abdominal haemocoel is impossible owing to the valve structure of the ostia (two lips inside) allowing only inflow into the heart (Wasserthal, 1982b). This type of ostium is the most widespread in insect orders. In Lepidoptera with a closed caudal heart end, the two-way ostia (one lip inside, one lip outside) allow haemolymph passage also from the heart into the abdominal haemocoel (Wasserthal, 1982b). During forward pulses, the haemolymph wave closes the ostia by pressing the inner ostial flaps against the overlapping heart tube. During backward pulses, the haemolymph is streaming against the anteriorly directed flap and opens it. As these ostia cannot prevent haemolymph from returning into the diastolic heart after systole, a pressure decrement between lumen of the diastolic heart and the abdominal haemocoel must exist for an efficient haemolymph transport. In butterflies (*Papilio* and *Caligo*) and moths (hawkmoths and giant silkmoths), the abdomen elongates periodically, always before the onset of backward pulse

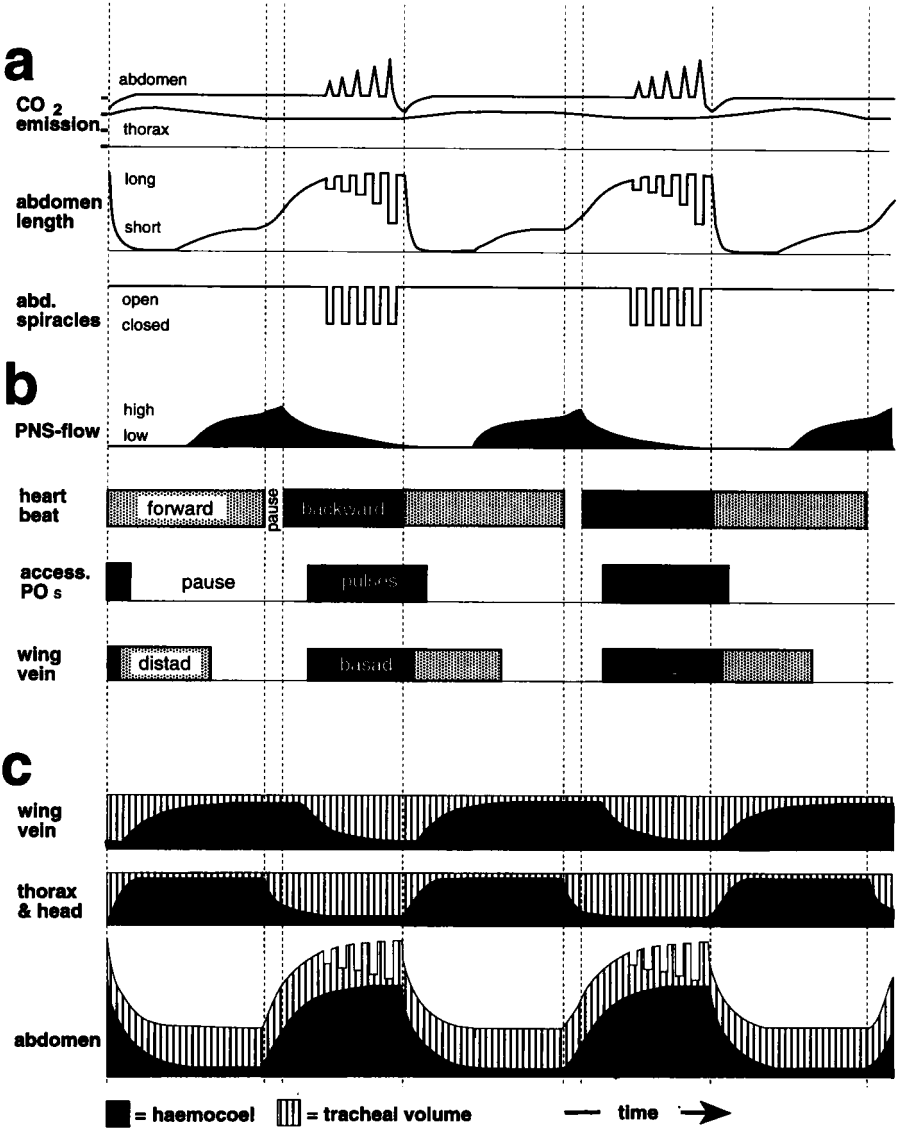

FIG. 20 (a–c) The interaction of coordinated discontinuous tracheal ventilation (a) with periodic haemolymph shift (b) in the resting giant silk moth *Attacus*, and their effects upon tracheal- and haemolymph volume of anterior and posterior body (c). (a) Bouts of peristaltic movements are superimposed on the slow periodic abdominal length changes in fully quiet individuals. Synchronized closing of the spiracles enhances the compressive effect and forces air to leave from the anterior abdominal spiracles: the corresponding CO_2-pulses are only detectable as part of the abdominal output, whereas the thorax shows continuous CO_2-emission with a slight increase

periods, continuing its length increase throughout backward beating and contracts again at the beginning of the forward pulse periods (Wasserthal, 1980, 1981). In *Attacus*, the abdomen performs an overall length increase also during the slow ventilatory movements during the second half of a backward pulse period. It is probable that this abdominal length increase (may be by relaxation or by tonus changes of the abdominal wall muscles) establishes the postulated more negative pressure in the abdominal haemocoel. The second prerequisite for efficient backward heart pulsations from the thorax into the abdomen is a sufficient haemolymph supply from the anterior body for refilling the diastolic heart. In the giant silk moth *Attacus*, the mechanism of a single backward pulse has been analysed, measuring the metachronous convective events by slightly heated microthermistors, stuck onto the tergites above the anterior and the posterior end of the abdominal heart, on top of the aortal loop, the mesotergal pulsatile organ (POII) and metatergal organ (POIII) (Figs 21 and 22). Additionally, high-speed cinematography has been used to observe these pulsatory systems through the descaled cuticle (Wasserthal, 1978). In Lepidoptera these tergal POs are pulsating intermittently (Wasserthal, 1976, 1980, 1982a). In silkmoths their pulse periods begin shortly after the start of the backward pulse period of the heart and stop as soon as haemolymph returns into the thorax after the onset of the forward heartbeat period. The POII discharges the sucked wing haemolymph via ostia directly into the aortal loop. Since the aortal ostia are positioned at the roof of the aortal loop, that means at a right-angle with respect to the aortal haemolymph flow, the inner

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during overall shortening of the abdomen. This coincides with change of heartbeat from backward to forward. During relaxation of the abdomen, haemolymph flow begins in the PNS. Further, probably active length increase induces heartbeat pause and following backward beating. (b) Coordination of heartbeat reversal with discontinuous haemolymph flow in the PNS, intermittent pulse activity of the accessory thoracic pulsatile organs and the resulting centrifugal and centripetal haemolymph flow in the wing veins. Haemolymph flow through the PNS, beginning in the second half of forward pulse period, is strongest during heartbeat pause and decreases rapidly during backward pulse period. The accessory mesothoracic pulsatile organs begin their pulse activity some seconds later than the backward pulse periods. Since at this time thorax is largely emptied of haemolymph, the POs suck haemolymph from the wing veins. They stop their pumping activity with some retardation after onset of forward beating of the heart, thus enabling a phase of lateral circulation in the thorax along the wing bases. After cessation of the PO activity, haemolymph is flowing centrifugally into the blindly ending wing vein due to the suction force of the wing tracheae (see below). (c) Substitution of periodically shifted haemolymph volume by tracheal volume in the anterior body and wings with largely constant total volume owing to uncompliant exoskeleton. In contrast, the total volume of the abdomen is periodically changed by haemolymph shift. While tracheal ventilation of the anterior body with its appendages is thus performed by this volume substitution, abdominal tracheal ventilation is performed by bouts of compression movements of the abdomen wall. Combined from Wasserthal, 1976, 1980, 1981 and Fig. 19.

ostial flaps must be closed by the aortal haemolymph pressure no matter whether the flow direction is forward or backward. The smaller POIII is morphologically not directly connected to the aorta; it discharges the haemolymph sucked from the hindwings via the metathoracic haemocoel into the aorta.

From the recorded consecutive convective events of the heart, aorta and POII (Fig. 21) a metachronic order of pumping is reconstructed (Fig. 22). At the beginning of each backward pulse, the first (weak) convective effect is perceivable at the top of the aortal loop (Fig. 21, step 1) and practically at the same moment but more pronounced at the second heart segment (step 2). It is followed by a convective pulse of the POII (step 3), a second marked cooling at the aortal loop (step 4) and a second weak effect at the second heart segment (step 5). These convective events represent firstly the beginning of the systolic peristaltic wave, which is initiated by the anterior pacemaker situated between aorta and the second heart segment (Tenney, 1953) (effect 1 and effect 2 practically simultaneously), and secondly the refilling of the anterior heart by the POII via aorta (4) during diastole (5). The duration of the backward pulse from the aorta (event 1) to the posterior end of the heart (event 6 at the 7th abdominal tergite) is 0.87 s (at the beginning of backward pulse period) to 1.1 s (at its end) at 23°C (Fig. 22). The pulse rate of the POIII is 1.5 to 1.8 times higher than the heart pulse rate. Its haemolymph pulse – damped by the metathoracic haemocoel – is entering the aorta mostly at moments different from the filling by the POII, so that it supplies haemolymph to the diastolic heart during the supplying pause of the POII. Thus, the backward haemolymph transport through the heart is facilitated by a system reminiscent of 'vacuum pumps arranged in series' with a hierarchical order: 1. Abdominal wall muscles: Generating the basal pressure gradient, 2. Heart: Transport between compartments, 3. Mesotergal PO and metatergal PO: Supply from periphery. In insects with accessory POs being morphologically not directly connected with the aorta, like butterflies and higher flies, the pulse frequency and activity periods exhibit a lesser degree of coordination with heart activity (Wasserthal, 1980 and unpublished results). During forward beating of the heart, PO-activity sucks haemolymph from the lateral thorax, maintaining a lateral circulation and distribution of haemolymph along the bases of the wing veins or in insect orders with cross-connecting wing channels a circulation through the wings as described by Arnold (1964).

5.4 A BACK PRESSURE VALVE BEHIND THE WAIST SERVES TO AUGMENT THE HYDRAULIC FUNCTION OF ABDOMINAL HAEMOLYMPH FOR ABDOMINAL VENTILATORY COMPRESSION

As a continuation of the vertical septum separating the anterior from the posterior part of the body at the level of the second abdominal segment, in Lepidoptera a horizontal plate of densely arranged fat body extends ventrally

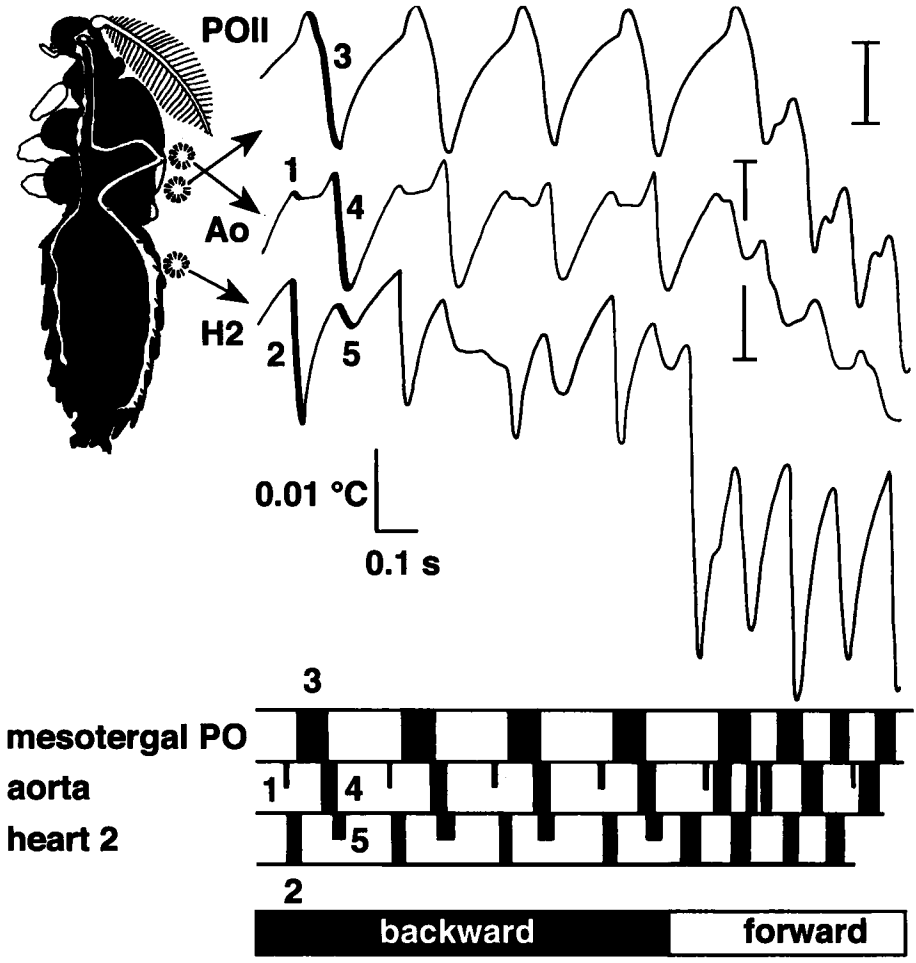

FIG. 21 Metachrony of convective events of the mesotergal pulsatile organ (POII), the aorta (Ao) and the heart (2nd tergite: H2) of a single backward heart pulse in *Attacus atlas*. Contact thermographs with three microthermistors 0.1 mm diam. Black bars and numbers indicate the moment and sequence of convective cooling events of the microthermistors heated to 1.7°C above ambient. The POII shows only one, however extended, convective cooling effect (3), representing the aspiration of wing haemolymph and consecutive discharge into the aorta. Aorta and heart perform two convective events representing systole (1 and 2) and refilling during diastole with haemolymph of the POII-discharge (4 and 5). See also Fig. 22.

and caudally into the abdomen (Fig. 22f). Together with the anterior portion of the ventral diaphragm, which fits well into the v-shaped 2nd and 3rd abdominal sternites, it prevents a haemolymph reflux through the PNS into the thorax during abdominal compression (Wasserthal, 1980, 1981). The periodic abdominal shortening as well as the brief abdominal ventilatory contractions, are not perceptible as convective effects within the haemocoel at any part of the anterior body in contrast to the observations in the honey bee (L. T. Wasserthal and W. Molsbach, unpublished). They occur as bouts with increasing amplitude during backward pulse periods of saturniid moths (Figs 19 and 20), during forward pulse periods of *Papilio* (Wasserthal, 1980) or as a single forced contraction at the very beginning of the forward pulse periods in *Caligo* (L. T. Wasserthal, unpublished). They ventilate only the abdominal air sacs. The tracheal system of the anterior part of the body is ventilated by slow compensatory volume changes due to the haemolymph shift.

5.5 DIFFERENCE OF SPIRACLE FUNCTION IN PUPAE AND ADULTS

While in pupae spiracle closing serves for a reduction of water loss by evaporation, in resting adult Lepidoptera spiracle closing has been observed only during compression movement at the abdominal segments concerned. As the peristaltic wave begins at the caudal end and proceeds to the anterior abdominal segments, an air stream must be directed towards the anterior abdomen. Although the tracheal system of at least the anterior abdominal segment communicates with the thoracic system, a corresponding pulsed outflow of heat-marked air or CO_2 from the thoracic spiracles is weak or not measurable at all (Wasserthal, 1981; Fig. 19b). The thoracic spiracles are generally more or less opened at rest and enable slow exchange of air according to the periodic compensatory volume changes visualized by a slightly increased CO_2-output during forward pulse periods (Fig. 19b) and a rise in air temperature at the metathoracic spiracle at that time (Wasserthal, 1981). The different function of this thoracic spiracle in Lepidoptera may also be deduced from its different morphology with an external valve (Nikam and Khole, 1989), different innervation and occurrence of an opener muscle instead of the closer muscle system of the other spiracles (Nüesch, 1956). Thus, spiracular closing in adult Lepidoptera helps in increasing the compressing effect of the abdominal

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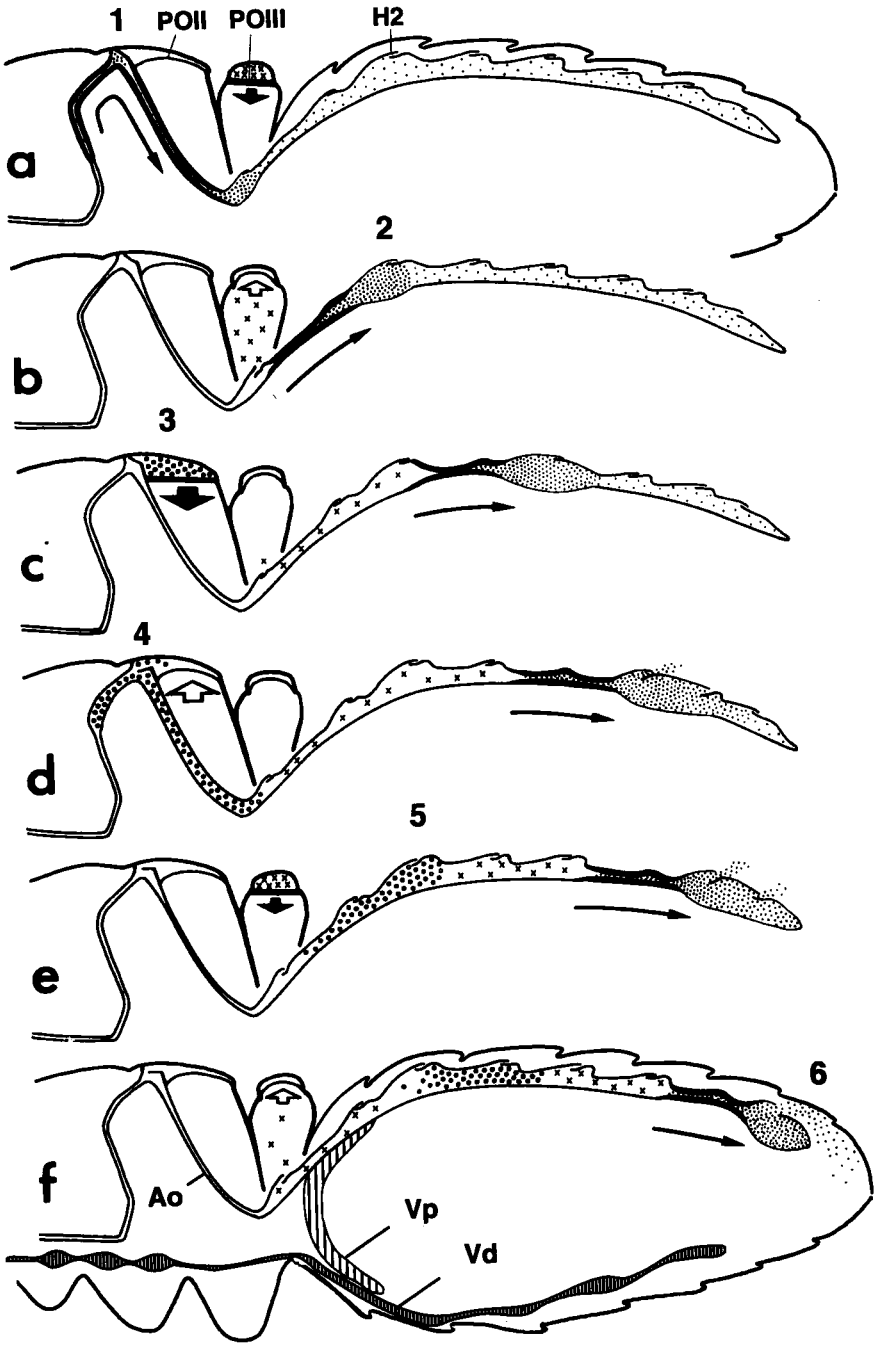
FIG. 22 Cooperation of mesotergal and metatergal pulsatile organs (POII and POIII) with the heart during a single backward pulse in the giant silk moth, *Attacus*. Reconstruction on the basis of contact-thermography (see Fig. 21) and high-speed cinematography. At the end of the peristaltic backward wave, the (dotted) haemolymph is leaving through the posterior ostia (6). Refilling of the diastolic heart via aorta comes from the POII (c–d: circles) and from the POIII, which beats at a rate 1.5 to 1.8 times higher than that of the heart (a–b and e–f: crosses). Ao = aorta, Vd = ventral diaphragm, Vp = valve plate of fat body.


1
POII
POIII
H2
a
2
b
3
c
4
d
5
e
6
f
Ao
Vp
Vd

ventilatory movements and produces an anterograde air flow. A similar spiracular activity may be performed by honey bees, where the spiracles of the first thoracic and abdominal (propodeal) segment behave in a different way at rest and also during ventilatory movements; they are probably producing a directed air stream during activity by segment-specific spiracular activity with an outflow through the propodeal spiracle (Bailey, 1954).

In adult insects with long periods of no CO_2-output, such as diapausing *Oryctes*, sleeping bees and those inactive adult insects from which a clear constriction phase in combination with discontinuous ventilation is described, a combination of both spiracular functions, water vapour retention during constriction and flutter phase and intensifying of tracheal ventilation by directing an air flow during compression movements might occur. However, the generally accepted water-saving function by constricted or fluttering spiracles has recently been questioned in adult hemimetabolous insects, because the water loss during the burst phase in *Romalea* grasshoppers is relatively low (1.9–13%) in relation to transcuticular evaporation and also because discontinuous ventilation is replaced by continuous random CO_2-release in dehydrated grasshoppers, when the demand for water retention is highest (Hadley, 1994). The variable temperature-dependent reaction of the respiration system in honey bees (Lighton and Lovegrove, 1990) and the transition from discontinuous to continuous gas exchange in pupae with increasing metabolism (S. K. Hetz and L. T. Wasserthal, in preparation), shows that adult insects and pupae are able to adapt their basic mechanism to changing requirements even at rest.

5.6 SPECIALIZED TRACHEAE AND AIR SACS AS SUCTION PUMPS FOR HAEMOLYMPH TRANSPORT

In most adult insects with a rather uncompliant exoskeleton of the anterior part of the body, a haemolymph shift into the abdomen is compensated by a volume increase of the anterior air sacs. In addition to the correlated slow inspiratory influence upon the tracheal system of the anterior body, another overlooked function of the distended tracheae is their function to suck haemolymph into their surrounding haemocoel as an antagonistic system to the muscular pumps. The sucking function was first demonstrated in the veins of *Attacus* wings (Wasserthal, 1982a). The haemolymph flows centrifugally and simultaneously in all wing veins towards their distal end at the beginning of the forward pulse period, when the accessory POs can no longer maintain their evacuation force upon the veinal haemocoel against the sucking force of the distended tracheae. As soon as sufficient haemolymph is available after the first forward pulses, the force of the tracheal system surpasses that of the concurring POs, which stop their pulsating activity after a delay (Wasserthal, 1976, 1982a; Fig. 20). These wing tracheae possess double-coiled taenidia (Fig. 23a), which enable their inflation to fill almost the entire

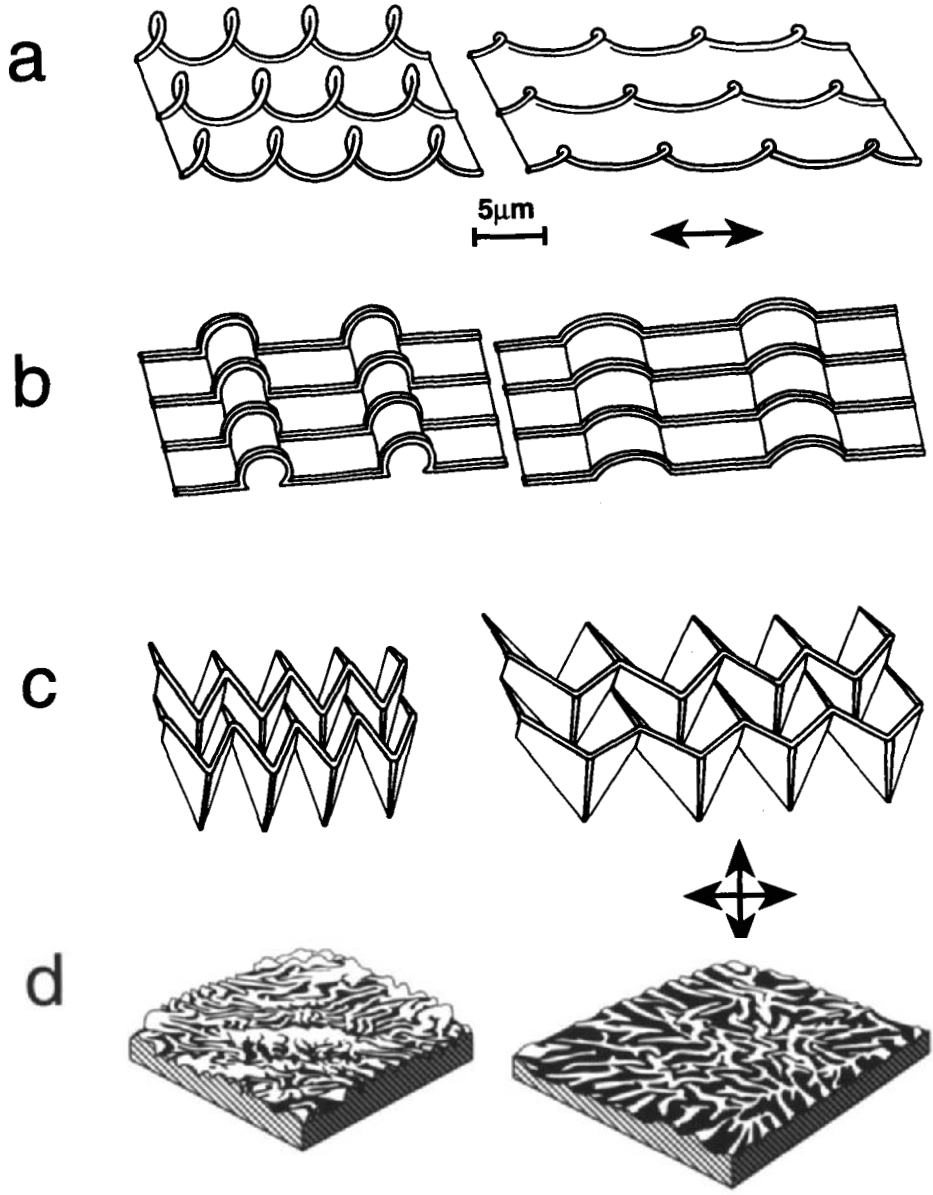


FIG. 23 Taenidial structures adapted for expansibility of specialized tracheae and air sacs. Left: low tension condition, right: high tension condition. (a) Double-coiled taenidium of wing trachea in *Attacus*. (b) Clamp-spring-double taenidium of the abdominal air sacs in the blowfly *Calliphora*. (c) Bellows-intima of abdominal air sac in the hawkmoth *Acherontia atropos*, a specific adaptation to the continuous abdominal pumping movements of this large moth. (d) Irregular plication pattern of elytral air sacs in the beetle *Goliathus*. This intima type is widely distributed among insects. Types (c) and (d) are extremely extensible within two directions.

veinal lumen during maximal evacuation of the vein haemocoel. In this distended condition they maintain their high tension until the return of haemolymph into the wing lacunae, when they attain the less distended round cross-shape. The wing tracheae of other Lepidoptera are also distensible and work in a similar way, but they have 'simple' spiral taenidia (hawkmoths), which reduces the degree of expansibility, or the spiral taenidium is less developed (butterflies). In both cases the distensibility is due to the intertaenidial surface, which is irregularly crinkled similar to the intima of the pupal tracheae (Fig. 5b). While the pupal leg is divided into two haemolymph lacunae by a longitudinal septum, which probably facilitates a circulation, in the adult leg a longitudinal air sac has substituted this septum and the minute pupal trachea. This air sac is attached to both lateral sides of the tibia and femur (Wasserthal, 1982b). In the less distended state the dorsal and ventral tracheal wall is stretched, separating two haemocoelic spaces. During haemolymph evacuation mainly the dorsal wall is extended into the haemocoel while at the ventral side, leg muscles, nerves and apophyses restrict the expansibility of the air sac. During haemolymph in- and outflow some circulation might occur and be superimposed upon the haemolymph oscillation.

In the scarabaeid beetles, the elytra contain chains of tracheal air sacs (Figs 16, 17). In the goliath beetle they are so distended that they form a polygonal mosaic. They contract if saline is allowed to invade into the haemocoel lacunae of a freshly severed elytron (Wasserthal, 1982b). In the living beetle the haemolymph periodically flows distally into the elytron during forward pulse period and centripetally into the body during backward pulse period of the heart (Fig. 14). In the rhinoceros beetle *Oryctes*, these periodic volume changes could be visualized by time lapse cinematography in individuals that were sleeping quietly under a microscope with their elytron made optically transparent and with a reference thermistor at the pygidium. The elasticity of the air sacs is facilitated by an irregularly buckled intima (Fig. 23d). After haemolymph has returned into the wing haemocoel, a phase of circulation occurs. Thus, in *Oryctes*, a haemolymph oscillation is combined with a short circulation phase. As the inflation–deflation process of the air sacs in the elytron is too slow to be recognized by the human eye, only the circulation of the blood cells has been observed by earlier authors (for literature see Arnold, 1964). Arnold, who suggested the occurrence of a circulation with inflow of haemolymph into the anterior wing veins (Costa, Subcosta, Radius and Media) and outflow through the posterior ones (Cubitus, Analis), also perceived irregular effects, which point to a wider distribution of periodic haemolymph shift in insect wings: 'Periodically in any insect, circulation in the wings may falter, reverse its direction of flow completely or in part, or stop entirely for short intervals. These periods of change tend to increase in frequency and duration with age, but they are normal occurrences at any age. They are unquestionably expressions of the unconfined nature of the circulatory system

as a whole, and of inefficiency on the part of the mechanism for wing circulation.' The latter conclusion can, however, not be accepted from our recent knowledge of a rather sophisticated supply system.

In contrast to the air sacs of the anterior part of the body, the abdominal ones must not only enable inflation but also resist collapsing during abdominal compression. They have evolved various plication patterns of their intima (Fig. 23b,c). Especially in Lepidoptera, Diptera and Hymenoptera, it seems that the air sacs are easily kept open, not only by negative pressure in the abdominal haemocoel, but also by capillary forces and adhesion.

5.7 FLEXIBLE OR FOLDABLE APPENDAGES WITH SPECIAL REQUIREMENTS FOR HAEMOLYMPH SUPPLY

In the anterior part of the body, which has a generally subatmospheric pressure, specific situations may demand a locally elevated haemolymph pressure, e.g. for extension of the tongue (galeae) in Lepidoptera and opening of the hindwings (alae) of beetles. Remarkably, the different structural preconditions of proboscis and alae require a haemolymph supply different from the other appendages (legs, antennae, elytra) of the adult, because they contain blindly ending lacunae with undistensible relatively small tracheae with a round cross-shape, which resists higher haemolymph pressures and bending without collapsing.

As the haemocoel of the lepidopteran tongue is divided by longitudinal septa into two or three lacunae becoming confluent at the tip (Eastham and Eassa, 1955), comparable to pupal legs, haemolymph supply should be performed here as a circulation. This circulation is possibly maintained or aided by the the stipital muscles. The stipital organs beside the bases of the galeae have so far been suggested to function as a valve to preserve pressure (Eastham and Eassa, 1955) or to generate an increased pressure in the proboscis for its extension, because they pulsate during extension (Schmitt, 1938; Bänziger, 1971; Krenn, 1990). It is an open question as to how both functions, local haemolymph supply and pressure generation for extension, are compatible with one another, assuming that circulation in the galeae is maintained during exercise of the intrinsic muscles.

The elastic outer venal cuticle along the transverse folds of beetle hindwings offers some compliance and is probably set under tension by periodic haemolymph reduction during backward beating (Fig. 17b). These elastic parts of the veins might suck haemolymph centrifugally like a pipette during forward beating of the heart (Fig. 17a). Scarabaeid beetles are known for their preflight abdominal pumping movements which visibly enlarge the abdominal air sac volume, one function of which is an increase of respiratory reservoir (Babak, 1921). A further effect of this pumping might be to displace most abdominal haemolymph from the abdomen into the anterior body by substituting the abdominal haemocoelic volume by air sac volume in order

locally to increase the haemolymph pressure for unfolding the alae. In this situation a prolonged anterograde transport in the dorsal vessel should be postulated. Conversely, the refolding of the hindwings in these beetles is a wearisome procedure, which might have its origin in the necessity to reduce the haemolymph content and to reinstall a negative haemocoelic pressure in the veins by retrograde heartbeat.

5.8 HEART ACTIVITY AND TRACHEAL VENTILATION DURING FLIGHT, PREPARATION FOR LOCOMOTION AND STRESS

While the circulation and respiration system of insects exhibits rather complex interactions even at rest, the adaptations to the requirements for flight must be expected to be much more sophisticated. Only few data exist about circulation, heart activity and ventilation in flying insects. From *Manduca* contradictory observations exist about heart activity. In free-flying moths only forward pulses with a rate of 120–180 pulses per min at T_{thorax} 38°C have been reported (Heinrich, 1970, 1971). During tethered flight alternating pulse frequencies have been recorded from the metathorax with impedance measurements (Tublitz, 1989): The pulses with higher frequency (which rose from about 40 min^{-1} at rest to about 55 min^{-1}) have been interpreted to be forward pulses and those with low frequency (which rose from under 10 min^{-1} to 20 min^{-1}, both at T_a 20–23°C) to be backward pulses. The pulse rates during flight appear to be relatively low and correspond to heart rates of resting *Sphinx ligustri*, a hawkmoth with similar size (Fig. 6). It is questionable whether the moths attained full flight activity under tethered flight conditions and whether the recorded changing pulse activity is due to heartbeat reversal or to the intermittent pumping activity of the metatergal pulsating organ (POIII) or some interference of both organs. The POIII is situated medially in the dorsal metathorax also in hawkmoths and can easily be overlooked (Brocher, 1919), while the aorta in this segment is located rather inaccessible more ventrally. The pulse rate of the POIII is independent of heart rate, generally higher, but can vary extremely under experimental conditions. In free-flying, fully intact and vital hawkmoths *Acherontia atropos* and *Hippotion celerio*, backward pulsations of the heart, which would have been visible by their heating effect, could never be recorded with microthermistors stuck upon the 2nd (abdominal) heart segment (L. T. Wasserthal, unpublished). In *H. celerio*, the forward pulse rate was raised from 32–48 min^{-1} at T_a 16.5°C to 149–176 min^{-1} at T_{thorax} 31–32°C (measured at the descaled cuticle at the top of the mesotergal aortal branch, for the duration of a dozen continuous measurements of 5–10 min each) confirming the data of Heinrich (1970). It looks as if during free flight the discontinuous oscillation events make way for a continuous pulse activity of the heart without any pulse pauses.

Some specific reactions of the circulatory and respiratory system can be

induced in resting insects by external stimuli, which normally might announce approaching predators. Some of these reactions can be interpreted as being preparations for running or flight activity. In the blow fly the rapid frequency phases have repeatedly been reported to be inhibited by optical and other stimuli (Medioni and Campan, 1967; Campan, 1972; Thon and Queinnec, 1976; Angioy, 1988). Because of the coincidence of walking activity with only slow beating phases of the heart, the cardiac responses were interpreted as preparatory to locomotor activity (Thon, 1980, 1982). However, as discussed above, the interpretation that the long pulse periods with low pulse rate are backward and the shorter periods with higher rate are forward pulse periods could not be reproduced in our *Calliphora*; it is just the reverse (Figs 11 and 12). Thus, sudden running activity should stimulate forward beating. During continuous running over minutes, however, periodic heartbeat reversals continue, but with higher pulse rate and shorter intervals (L. T. Wasserthal, in prep.). While the above authors concentrated their interest on the influence of light stimuli upon changes of heartbeat direction and frequency, we found that the immediate reaction of *Calliphora* upon light on and off, or vibrating stimuli, is to fully stop the heartbeat for several seconds up to half a minute, especially in the white-eyed mutant! Remarkably, in *Aedes* mosquitoes, ventilatory activity is briefly inhibited by similar stimuli (Miller, 1981).

In the giant silk moth, slight disturbances (vibrations, sudden shadow, tactile stimuli) provoke very efficient backward pulsations with especially low frequency, which are visibly connected with slow lifting of the forewings (L. T. Wasserthal, unpublished). A comparable spontaneous raising of the wings occurs prior to shivering in several species of hawkmoths. This behaviour is interpreted as a pre-flight decrease in the volume of the wing haemolymph, which means a reduction in wing weight and reduces the quantity of wing haemolymph driven by centrifugal forces into the distal veins. In resting *Acherontia atropos* disturbances including ultrasonic stimuli are responded to by a short interruption or alteration of the typically uniform abdominal pumping movements, often combined with abdominal length changes and heartbeat pauses (L. T. Wasserthal, unpublished). In *A. atropos* this may be the expression of a 'startling reaction', which in active (shivering or flying) moths evokes immediate cessation or alteration of wing beat with a predator-avoiding effect.

In the resting adult insect, the metabolic rate is higher in the abdomen than in the anterior body due to the concentration of fat body, digestive and reproductive organs and the muscle activity of body wall, heart, ventral diaphragm, intestine and gonadal tracts and it is understandable that the CO_2 output is higher here (Fig. 19). During locomotor activity, the thorax has the higher metabolism, and the respiratory system has to manage the removal of great amounts of CO_2 from the flight muscles. Autoventilation, which has been described in the synchronous muscles of locusts with 30% contractility,

may be less efficient in the asynchronous flight muscles of holometabolous adults with only 1% length change and with little deformation of the pterothorax (Weis-Fogh, 1967). As during flight haemolymph circulation seems to be rather continuous, it should have a negligible ventilatory effect upon the tracheal system. Thus, other efficient ventilation mechanisms have to be postulated for the respiratory gas exchange in most adult holometabolous insects during flight. In some insects as in the honey bee, the pumping abdomen may generate an airstream entering the first thoracic spiracles and leaving the second thoracic spiracle or other more posterior ones (Bailey, 1954). In diverse beetles with weak or no abdominal ventilatory movements during flight, air supply may be supplemented by 'draught ventilation' through large, low-resistance, primary tracheae (Miller, 1966).

6 Concluding remarks

General blood pressure generation and transport activity, which in a closed circulatory system are both performed mainly by one single pump, the heart, in insects are carried out by different organs. The muscles of the (mostly abdominal) body-wall serve for basic pressure generation, and the dorsal vessel, ventral diaphragm and accessory pulsatile organs serve for haemolymph transport. Directed haemolymph transport by contractions of the body wall alone can be brought about only if a pressure decrement can be built up. This is not possible in pupae and larvae with a single compartment. Only in the emerging pharate adult and especially during wing inflation, can abdominal wall compression transport haemolymph directly and effectively through the waist into the expanding wings. In the fully developed adult, the abdomen wall muscles can still exert negative and positive pressure upon the abdominal haemocoel and tracheal system, but their influence upon the haemocoel and tracheal system of the anterior body is less direct and mediated by the ventral sinus and/or heart in different ways within different insect orders. Owing to the narrow waist, tightened by organs, passing through it and sealing tissues, a direct anterograde haemolymph passage from the abdomen to the thorax is difficult or impossible in most holometabolous adults. Only via the longitudinal tracheal trunks, can abdominal pressure be propagated as a directed air stream into or from the anterior body as in some Hymenoptera. The small quantities of haemolymph in the adult are better transported and directed by smaller pumps in cooperation with the antagonistic suction force of the distended elastic tracheae and air sacs especially within the anterior part of the body and its appendages. But in adults the basic haemocoelic pressure is not independent of the tension of abdomen (body-wall) muscles and, similarly, as in Lepidoptera, a basic pressure gradient is a prerequisite also for efficient

haemolymph transport in dorsal vessels with caudal openings as in some flies and beetles.

Within an open uniform haemolymph system surrounded by a rather uncompliant exoskeleton, as in pupae, most muscle activity is propagated as a pressure effect. It is therefore often difficult to identify or localize the motor responsible for a specific measured pulse. An attempt has been made to generalize most pressure changes as 'coelopulses' for different instars and various insect orders (Slama, 1988, 1991). Quite different conditions exist, however, in the three main developmental stages. Whereas the larvae possess high haemolymph pressure and volume in combination with a soft and elastic outer exoskeleton, pupae have a sclerotized and more uncompliant exoskeleton with a high haemolymph volume but a low haemolymph pressure. Adults have a low haemolymph pressure and volume but a high tracheal volume in a partitioned exoskeleton with a more or less uncompliant anterior part of the body and a partly compliant abdomen. These drastic differences are the basis of different supply mechanisms which evolved as adaptations to the often fundamentally different 'job' and way of living of phlegmatic feeding larva, passive re-embryonalized pupa and the lightweight, flight-adapted adult.

The partitioning of the adults in combination with the periodic haemolymph shift allows the local reduction or accumulation of the small haemolymph volume for hydraulic purposes in the compartment where it is needed. The slow haemolymph shift by heartbeat reversal produces in both compartments a reciprocal effect upon the tracheal volume, so that the anterior system inspires when the abdominal one expires and the converse, if the spiracles are open as in the fly *Calliphora*. This mechanism of ventilation by periodic haemolymph shift (may be by heartbeat reversals as in flies, beetles and Lepidoptera or by discontinuous circulation with heart pauses and intermittent backflow through the PNS as in *Apis*) is very inconspicuous and has therefore been overlooked to date. As some adult insects keep their spiracles closed during several haemolymph oscillation periods, the question arises, as to whether further effects are produced by this haemolymph shift. The indirect haemolymph transport by the suction force of the distended air sacs also functions with closed spiracles. The partitioned body allows the generation and maintenance for a while of different pressure conditions in both haemocoels and, with the spiracles closed, also in the tracheal system. It should be considered whether the periodic haemolymph pressure differences are also utilized for optimizing the transport of O_2 from the tracheoles into the consuming cells or for the transition of the dissolved CO_2 into the tracheal lumen. The insect respiratory system without the existence of respiratory pigments and direct tracheal O_2 supply to the tissues, still has the capacity to use the haemolymph, at least partly, as a specific pathway and store for CO_2 instead of returning it from the tissues directly into the finest branches of the supplying tracheae. A CO_2

storing function for haemolymph is confirmed in pupae and resting adults with long constriction phases and bursting CO_2 emission. A mechanism of efficient CO_2 transport should have also been evolved in powerful adults, especially during high activity. It is probable that the CO_2 released from the tissues into the haemolymph, could be discharged from the haemolymph into those tracheae or air sacs facing the haemolymph directly and with a great surface area, preferentially near the spiracles. The retrogradely transported haemolymph might discharge its CO_2 via abdominal air sacs, thus partly relieving the thoracic tracheal system from this 'duty'. Such a mechanism would imply a kind of respiratory gas circulation in the insect. It might be well established in those adults with large air sacs in the waist (metathorax and anterior abdomen) but also in adults of other insect orders with numerous abdominal air sacs. Examples (that have been interpreted as suction ventilation in adult insects, such as in ants, may prove to be generated by haemolymph shift combined with slow abdominal volume changes. In insect orders with a variable shape of the ventilating abdomen, the moment of maximal accumulation of haemolymph in the abdomen is often utilized for abdominal tracheal compression.

The aim of this article has been to evaluate the knowledge of circulation and tracheal ventilation mechanics. While competent reviews exist about circulation and respiration (see introduction) almost no attempts can be found in the literature to integrate the interactions of both supplying systems. In addition, in modern experimental work, both aspects have generally been treated separately. Therefore this review is based largely upon our own data and figures which have been elaborated specifically to visualize the interdependence of both systems. The desire to formulate conclusions has involved the risk of not fully taking into account the enormous variability and flexibility of insect adaptations, despite an attempt to concentrate upon representatives of the four most 'successful' and extensively examined holometabolous insect orders. There is still a huge gap of knowledge concerning circulation and respiration mechanics in insects, especially in active adults under physiological conditions. The development of miniaturized sensors for more precise and non-invasive measurements may contribute to a better understanding of the sophisticated open haemolymph system and tracheal system in insects.

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