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Mini-Review

Insect Storage Proteins: Gene Families and Receptors

N. H. HAUNERLAND*†

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The accumulation and utilization of storage proteins are prominent events linked to the metamorphosis of holometabolous insects. Storage proteins are synthesized in fat body, secreted into the larval hemolymph and taken up by fat body shortly before pupation. Within the pupal fat body, these proteins are initially stored in protein granules, and later proteolytically broken down to supply amino acid resources necessary for the completion of adult development. Most, but not all storage proteins belong to a superfamily of hexameric larval serum proteins that are evolutionarily related to hemocyanin. This article reviews the classification of these proteins, based on their amino acid sequences, and the current knowledge of the receptors that mediate their selective uptake into pupal fat body. Copyright © 1996 Elsevier Science Ltd

Storage protein Hexamerin Arylphorin VHDL Receptor

INTRODUCTION

Munn *et al.* (1971) described an abundant serum protein from *Calliphora erythrocephala* larvae which, shortly before pupation, was taken up by fat body tissue. Since the protein accumulated in dense protein granules, it was called storage protein, indicating its proposed function as an amino acid reserve for the production of adult proteins. Subsequently, similar proteins were found in other dipteran and many lepidopteran species. Because of their high content of aromatic amino acids these storage proteins were classified as arylphorins.

Over the years much information has been gained on the structure and distribution of these proteins. Arylphorins are large hexameric proteins composed of 80 kDa subunits. They were also discovered in other insect orders, including hemi-metabolous species. Furthermore, structurally similar hexameric proteins, collectively called hexamerins, were found that lacked the high content of aromatic amino acids. Several excellent reviews have been written about the composition, distribution and properties of hexameric storage proteins (Telfer and Kunkel, 1991; Kanost *et al.*, 1990), which summarize the knowledge accumulated up to 1990. Since then, dramatic progress has been made both on structural and functional aspects of storage proteins. Several of these proteins have been sequenced, shedding light on their structural relationships. In addition, structurally unrelated proteins have been discovered in some insect species which may be functionally equivalent to hexameric storage proteins. In the first part of this review, the different families of storage proteins are discussed and compared with other related proteins. The term "storage protein" implies uptake from the hemolymph and storage in fat body tissue. There, storage proteins appear to serve as a storage pool for the amino acid resources needed later in development. Whatever the ultimate fate of the proteins or their amino acid constituents may be, the uptake into fat body is common to all true storage proteins and therefore central to their function. This process has been studied intensively in recent years. The second part of this review is focused on the current advances in the elucidation of the uptake process of storage proteins.

STORAGE PROTEIN FAMILIES

The different classes of storage hexamers were comprehensively described in the review of Telfer and Kunkel (1991). These authors covered only hexameric proteins,

*Department of Biological Sciences, Simon Fraser University, Burnaby, B.C. V5A 1S6, Canada.
†Author for correspondence: Tel. (604) 291-3734; Fax (604) 291-3496; e-mail: haunerla@sfu.ca

including, but not limited to storage proteins. Such proteins have mostly been studied in Diptera and Lepidoptera, but more recently also in other insect orders. All dipteran species possess a protein rich in aromatic amino acids (>15%) and methionine (>4%). While different names have been used in the past for this protein, e.g. calliphorin (Munn and Greville, 1969) or larval serum protein 1 (LSP-1, Wolfe *et al.*, 1977) it is now called arylphorin, to describe its perceived function as carrier of aromatic amino acids. The second storage hexamer common to Diptera has a much lower content of aromatic amino acids and methionine; an example is LSP-2 from *Drosophila* (Roberts and Brock, 1981). Two common lepidopteran storage proteins, in contrast, are rich in either aromatic amino acids or methionine, but not both. Again, the name arylphorin is used for proteins with more than 15% tyrosine and phenylalanine content, while the second protein is referred to as "methionine-rich storage protein". Additional hexamerins have been described in some lepidopteran species, including several juvenile hormone suppressible proteins from *Trichoplusia ni* (Jones *et al.*, 1990, 1993) and *Galleria mellonella* (Memmel *et al.*, 1994), and a riboflavin binding hexamer (Silhacek *et al.*, 1994; Magee *et al.*, 1994). While all these proteins share similar subunit structures and developmental profiles in larvae, their uptake, storage and utilization has not always been established, and they may, therefore, not all be "storage proteins". On the other hand, at least one lepidopteran family possesses a storage protein that is structurally unrelated to hexamerins (Haunerland and Bowers, 1986; Jones *et al.*, 1988; Greenstone *et al.*, 1991); this very high density lipoprotein (VHDL) of Noctuids is, like arylphorin, synthesized mostly in last instar larvae, taken up rapidly by fat body prior to pupation, deposited in protein granules and later hydrolyzed (Wang and Haunerland, 1992). The hexameric structure is therefore neither sufficient nor necessary for proteins that function as storage proteins.

Storage proteins have also been found in other insect orders; arylphorins as well as other hexamerins were described in bees (Shipman *et al.*, 1987), ants (Wheeler and Martinez, 1995), beetles (DeKort and Koopmanschap, 1994), but also in the hemimetabolous locusts (DeKort and Koopmanschap, 1987) and cockroaches (Duhamel and Kunkel, 1983; Jamroz *et al.*, 1996). In most cases, little is known about their utilization during development, but evidence exists that at least arylphorin is taken up by pupal fat body in all holometabolous species. On the other hand, a non-hexameric protein has been found in pupal fat body of some coleopteran species that store aromatic amino acids (Delobel *et al.*, 1992).

While the early classification of storage proteins was based on their amino acid composition, many proteins have now been sequenced, giving a more reliable basis for their classification. All hexamerins show a clear homology to arthropod hemocyanins, and it is obvious that these proteins are evolutionarily related (Willot *et al.*, 1989). Hemocyanins are copper-containing, oxygen-binding proteins found in chelicerates and crustaceans, which also form multimeric structures from 80 kDa subunits. Beintema *et al.* (1994) have recently analyzed this protein superfamily in detail and proposed, based on multiple sequence alignments, that the insect hexamerins evolved from a common hemocyanin precursor. Since the tracheated insects generally have no need for oxygen transporting hemolymph proteins, the oxygen binding function may have been lost and the protein may have assumed different functions within the insect. By comparing the sequence identities within the insect hexamerin group, it was found that the hexamerins form four distinct groups which have evolved separately, namely (1) lepidopteran methionine rich proteins, (2) lepidopteran arylphorins, (3) certain lepidopteran juvenile hormone-suppressible proteins and (4) the dipteran storage proteins. This grouping does not correlate well with the classification that was based on amino acid compositions and it does not include several recently sequenced proteins. For a better understanding of the different storage proteins described above, it is helpful to correlate both functional aspects and structural data. However, it is difficult to compare the reported sequence homologies directly because different methods were used for sequence alignment. For the structural comparison given in this review, all currently known storage protein sequences were aligned with the ClustalW multiple alignment program (Fig. 1). To determine the homologies between individual proteins, their sequences were pairwise aligned using the ALIGN algorithm and their amino acid identity determined (Table 1). Sequence similarities were also calculated; these may be more important parameters for a classifications of the proteins since conservative substitutions tend to maintain the overall protein structure.

Arylphorins

Diptera Numerous studies have been carried out to elucidate the physiological functions of the dipteran arylphorin (for reviews, see Scheller, 1983). The protein is synthesized in fat body of larvae in the penultimate and ultimate larval stage, and secreted into the hemolymph where it accumulates in extremely high concentrations. When the insect begins wandering, arylphorin biosynthesis stops; instead, the protein is gradually taken up into pupal fat body, where it is stored in protein granules. Although detailed studies on different regions of fat body have not been carried out, there is evidence that storage protein granules are more abundant in the posterior region of the fat body. During the development of the pharate adult, much of the arylphorin is proteolytically destroyed and its constituent amino acids can be found in the newly formed cuticle. The arylphorin found in dipteran species has been sequenced from *Calliphora vicina* (Naumann and Scheller, 1991). The protein shows similar homologies to all other hexameric storage proteins, forming a separate hexamerin (Table 1).

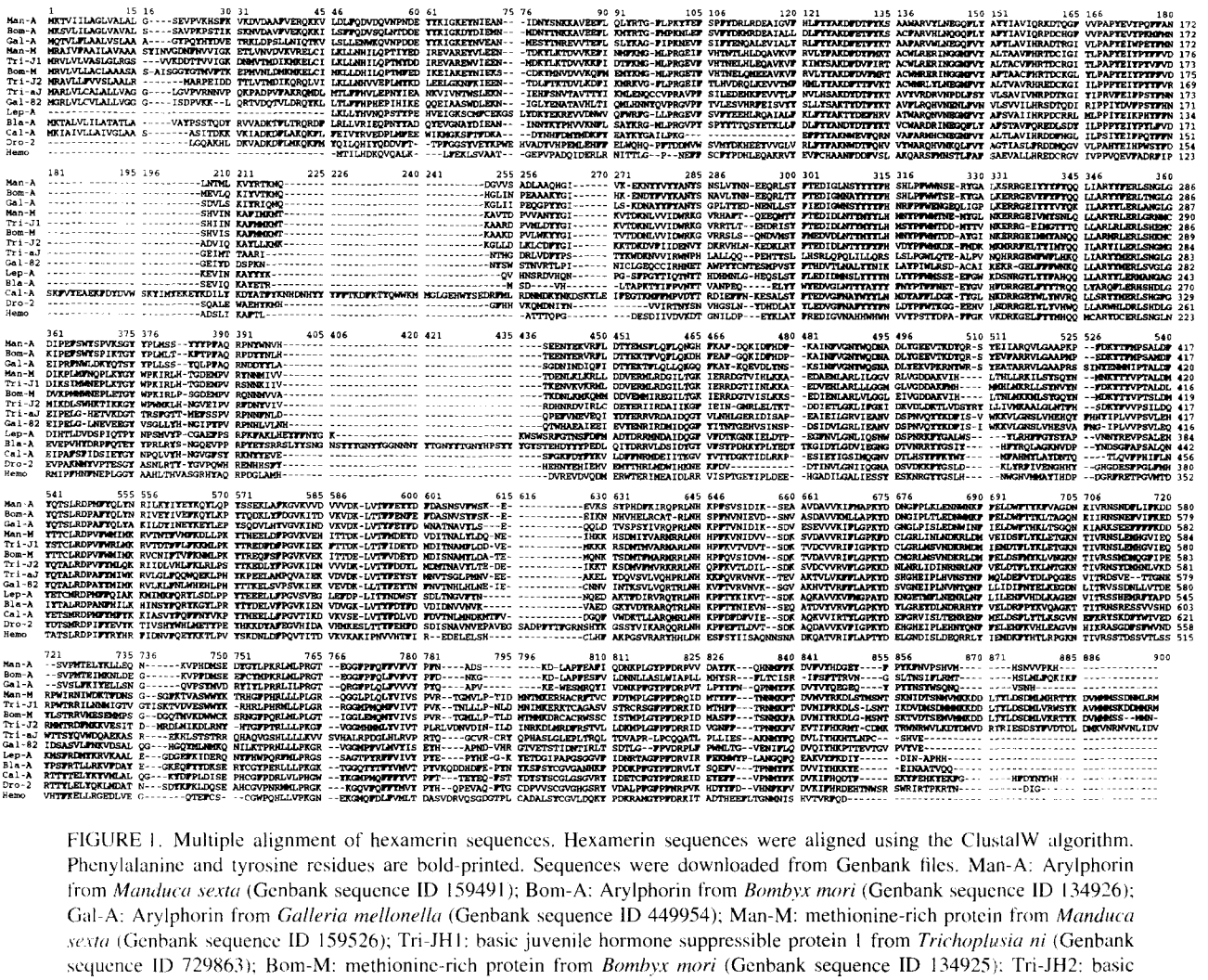

FIGURE 1. Multiple alignment of hexamerin sequences. Hexamerin sequences were aligned using the ClustalW algorithm. Phenylalanine and tyrosine residues are bold-printed. Sequences were downloaded from Genbank files. Man-A: Arylphorin from *Manduca sexta* (Genbank sequence ID 159491); Bom-A: Arylphorin from *Bombyx mori* (Genbank sequence ID 134926); Gal-A: Arylphorin from *Galleria mellonella* (Genbank sequence ID 449954); Man-M: methionine-rich protein from *Manduca sexta* (Genbank sequence ID 159526); Tri-JH1: basic juvenile hormone suppressible protein 1 from *Trichoplusia ni* (Genbank sequence ID 729863); Bom-M: methionine-rich protein from *Bombyx mori* (Genbank sequence ID 134925); Tri-JH2: basic juvenile hormone suppressible protein 2 from *Trichoplusia ni* (Genbank sequence ID 729864); Tri-aJH: acidic juvenile hormone suppressible protein from *Trichoplusia ni* (Genbank sequence ID 125066); Gal-82: juvenile hormone suppressible protein from *Galleria mellonella* (Genbank sequence ID 156154); Lep-A.: Arylphorin from *Leptinotarsa decemlineata* (Genbank sequence ID 556786); Bla-A: Arylphorin from *Blaberus discoidalis* (Genbank sequence ID 951139); Cal-A: Arylphorin from *Calliphora vicina* (Genbank sequence ID 288282); Dro-2: larval serum protein 2 from *D. melanogaster;* sequence obtained from Naumann and Scheller, 1991; Hem: hemocyanin from *Eurypelma californicum* (Genbank sequence ID 122799).

TABLE 1. Sequence similarities in the hexamerin superfamily. Sequence identities and similarities were determined by pairwise alignment using ALIGN. Identities are shown below, similarities above the diagonal line. Abbreviations and sequence sources as in Fig. 1

	Man-A	Bom-A	Gal-A	Man-M	Tri-J1	Bom-M	Tri-J2	Tri-aJ	Gal-82	Lep-A	Bla-A	Cal-A	Dro-2	Hemo
Man-A	-	90.0	85.5	68.8	68.3	68.3	69.7	69.5	69.4	66.6	64.8	64.4	65.9	63.5
Bom-A	66.6	-	83.3	68.5	66.7	67.7	69.5	68.0	67.1	67.2	63.2	65.4	65.6	61.8
Gal-A	54.2	49.9	-	69.7	67.1	67.1	70.0	66.6	69.2	68.0	63.0	63.8	65.8	62.3
Man-M	31.8	30.3	29.7	-	93.6	87.0	78.1	65.1	64.5	61.0	62.2	66.4	62.5	58.5
Tri-J1	29.0	28.9	27.9	71.2	-	66.2	78.5	67.5	66.0	61.0	59.5	65.2	62.4	59.2
Bom-M	31.4	28.0	30.0	69.5	91.8	-	78.5	65.9	67.0	60.5	60.0	65.2	64.5	58.6
Tri-J2	33.8	32.2	31.7	44.9	44.1	47.5	-	67.8	68.0	63.7	60.5	65.3	63.3	58.6
Tri-aJ	26.7	26.1	27.0	26.0	23.8	25.1	27.1	-	78.4	65.4	64.1	63.6	64.0	62.2
Gal-82	26.2	27.4	28.6	26.2	25.0	25.7	29.5	45.0	-	66.8	63.8	64.2	65.4	62.5
Lep-A	30.5	29.1	31.0	28.5	27.4	28.2	30.4	26.8	28.7	-	67.0	62.6	66.9	63.7
Bla-A	32.3	29.8	30.8	29.3	28.0	29.0	29.2	21.6	22.9	34.9	-	65.0	62.4	63.0
Cal-A	27.8	25.7	29.8	25.3	23.1	27.6	27.5	22.1	25.8	28.6	27.1	-	62.6	56.9
Dro-2	30.5	28.1	28.5	22.5	24.3	24.3	26.3	21.7	25.3	30.7	31.0	31.0	-	60.5
Hemo	24.7	25.1	24.8	23.6	23.7	25.0	27.2	21.6	27.2	26.8	27.4	23.4	25.3	-

Lepidoptera The overall characteristics in lepidopteran arylphorins resemble those of the dipteran proteins, but several important differences exist. Lepidopteran arylphorins have a similar percentage of aromatic amino acids, but are relatively low in methionine. The sequence identities between those lepidopteran arylphorins sequenced (*Manduca sexta,* Willot *et al.*, 1989; *G. mellonella,* Memmel *et al.*, 1992; *Bombyx mori*, Fujii *et al.*, 1989) is between 50 and 70%, but they are much less homologous to the dipteran arylphorin. While arylphorin is synthesized at high rates in fat body of last instar larvae and released into the hemolymph, evidence exists that the protein is expressed at a low rate throughout larval life (Ray *et al.*, 1987; Webb and Riddiford, 1988), and also in the gonads of adult insects (Miller *et al.*, 1990; Kumaran *et al.*, 1993). Nevertheless, high hemolymph concentrations are found only during the last larval instar and re-absorption into fat body begins in prepupae. In most cases, arylphorin is not completely removed from the hemolymph, possibly due to its high concentration (Haunerland *et al.*, 1990). In detailed studies the uptake and accumulation into fat body has been biochemically and electron microscopically documented in *Hyalophora cecropia* (Tojo *et al.*, 1978), *B. mori* (Tojo *et al.*, 1980) and *Helicoverpa zea* (Wang and Haunerland, 1991, 1992). Arylphorin has been positively identified in crystalline protein granules in fat body and it appears that these granules are gradually, but not completely broken down during the pupal stage; in fact, many granules have been detected in adult fat body and it has been suggested that arylphorin may also serve as an amino acid source for yolk protein production (Wang and Haunerland, 1991).

As for the place of synthesis and storage, most studies have not attempted to distinguish between different regions of fat body. Detailed studies have only been performed in *H. zea*, where, aided by a colored storage protein (VHDL, see below), a clear distinction could be made between the place of synthesis and storage (for a review, see Haunerland and Shirk, 1994). Arylphorin is synthesized in the larval fat body that is found peripherally, between the outer muscle layer and the cuticle of 5th instar larvae. This white tissue, however, does not sequester arylphorin, instead the protein is actively taken up by newly formed fat body tissue that is located centrally, within the body cavity, surrounding the gut. From electron micrographic studies it is now clear that this perivisceral fat body persists through the pupal stage and develops into the adult fat body; the peripheral fat body, however, decays.

Coleoptera Arylphorin-like proteins have been identified in a number of coleopteran species, including the mealworm *Tenebrio molitor* (Delobel *et al.*, 1992) and the Colorado potato beetle, *Leptinotarsa decemlineata*. The latter protein was recently sequenced (DeKort and Koopmanschap, 1994). It is rich in aromatic amino acids, as other arylphorins, but structural similarities with arylphorin from other families are not greater than with the remaining hexamerin classes (Table 1). Relatively little is known about its developmental profile, but it appears that the protein is also stored in granules of pupal fat body.

Dictyoptera In *Periplaneta americana*, two arylphorin-like molecules have been identified (Duhamel and Kunkel, 1983). One of these is present throughout all life stages, while the other one is found mostly in larvae. No evidence exists that points towards the uptake and storage in fat body, and the physiological role of these proteins has remained unclear. Recently, a related protein has been cloned and sequenced from *Blaberus discoidalis* (Jamroz *et al.*, 1996). The protein is similarly homologous to all other hexamerins, including the arylphorins from other families (Table 1).

LSP-2 from Diptera

The second storage protein known in Diptera is present in smaller amounts than arylphorin, but it is similar in its developmental profile. Apparently, this protein is also taken up into fat body, but its ultimate fate is not known. This protein may play an important role in the adult insect, as suggested by the fact that its expression resumes after adult eclosion (Benes *et al.*, 1990). LSP-2 from *D. melanogaster* is the only protein of this group that has been sequenced (S. Mousseron; cited in Naumann and Scheller, 1991); its sequence homology to dipteran arylphorin and to all other hexamerins is similar and relatively low, indicating no close evolutionary relationships between LSP-2 and any other hexamerin (Table 1).

Methionine-rich storage proteins from Lepidoptera

Methionine-rich proteins contain more than 4% methionine. In contrast to arylphorin, these proteins are not glycosylated. They have been found in several, but not all lepidopteran species investigated. In some species, e.g. *M. sexta* and *H. cecropia*, two or more isoforms of methionine-rich proteins have been found (Wang *et al.*, 1992; Tojo *et al.*, 1978). Their biosynthesis in fat body appears to commence later than the synthesis of arylphorins, towards the end of the last larval stage. Then, methionine-rich protein disappears completely from the hemolymph and is taken up by pupal fat body. While its deposition in granules has not been studied, Pan and Telfer (1992) demonstrated that its concentration in the fat body does not significantly decrease during the pupal stage. It is interesting to note, however, that, in contrast to all other classes of storage proteins and hexamerins, methionine-rich proteins, at least in *M. sexta*, are much more abundant in female than in male larvae (Ryan *et al.*, 1985). Sequence comparison indicates that two basic juvenile hormone-suppressible hexamerins described in *T. ni* (Jones *et al.*, 1993) also belong into this group (Beintema *et al.*, 1994). The identity between the methionine-rich hexamerins is relatively high, between 44 and 70% (Table 1). In contrast, identity with other lepidopteran and dipteran hexamerins is less than 34%. Since the

two basic juvenile hormone suppressible storage proteins from *T. ni* appear to be the equivalent to the methionine-rich storage proteins in other lepidopteran species, it is likely that the latter proteins are suppressed by juvenile hormone in other species as well. Indeed, it has been shown that the methionine-rich proteins of *M. sexta* appear only after the juvenile hormone titer declines in the final larval instar (Webb and Riddiford, 1988; Corpuz *et al.*, 1991).

Juvenile hormone-suppressible proteins

The expression of storage proteins is mostly confined to the last larval instar, a period where juvenile hormone titers are low, and it has long been suspected that the hormone prevents the production of storage proteins. Indeed, juvenile hormone has been shown to suppress the expression of some, but not all storage proteins. In addition to the above mentioned basic juvenile hormone-suppressible proteins, an acidic hexamerin has been characterized in *T. ni* (Jones *et al.*, 1990); amino acid composition, sequence homology and juvenile hormone-suppression suggest that a recently cloned hexamerin from *G. mellonella* (LSP-82, Memmel *et al.*, 1994) is an analogous protein. These acidic juvenile hormone-suppressible proteins are highly homologous to each other, but much less to all other hexamerins (Table 1). At this point, little is known about their developmental profiles and fate and whether they function as storage proteins.

Riboflavin-binding proteins

In spite of structural similarities to other hexameric storage proteins, it is known that the riboflavin-binding hexamerins found in *H. cecropia* (Magee *et al.*, 1994), *G. mellonella* (Silhacek *et al.*, 1994) and *Heliothis virescens* (Miller and Silhacek, 1992) are not true storage proteins. Although they are expressed strongly only in the last larval stage, they are not actively sequestered by fat body before pupation and do not accumulate in pupal fat body. Their hemolymph concentration diminishes rapidly during the pupal-adult eclosion, but it is not known whether these proteins are simply hydrolyzed or taken up by fat body, ovaries, or any other tissue. These proteins are glycosylated and appear to contain a high concentration of histidine and arginine. To date, no sequence information is available for these proteins.

VHDL

A non-hexameric storage protein has been characterized in the corn earworm, *Helicoverpa zea* (Haunerland and Bowers, 1986) and other Noctuid species (Jones *et al.*, 1988; Greenstone *et al.*, 1991). This protein, a dimeric or tetrameric complex of 150 kDa subunits, is characterized by two unusual properties: it is colored blue, due to non-covalently bound biliverdin, and it is a very high density lipoprotein (VHDL) with approx. 10% lipid. It is synthesized in peripheral fat body of last instar larvae, and accumulates in high concentrations in hemolymph, before it is specifically taken up by perivisceral fat body prior to pupation. Within the fat body, it can be found in protein granules, but it is apparently proteolytically digested within a few days. Its amino acid composition is not different from average proteins and thus it appears that this protein bears no relation to the hexameric storage proteins described above; however, sequence information is not yet available. VHDL is the only non-hexameric larval serum protein known so far that clearly functions as storage protein. In the hymenopteran species *Apis mellifera* (Shipman *et al.*, 1987) and *Camponotus festinatus* (Wheeler and Martinez, 1995) a VHDL with similar lipid and apoprotein structure, without the colored chromophore has been reported, but the developmental profile has not been established.

Tyrosine-Rich proteins

Tyrostaurins and related tyrosine-rich storage proteins are proteins with up to 27% tyrosine found in coleopteran fat body (Delobel *et al.*, 1992). Their synthesis and accumulation in fat body occurs shortly before pupation, and these proteins are deposited in protein granules of pupal fat body. Later in development these granules are partially broken down, suggesting that the tyrosine residues of tyrostaurins are used for the biosynthesis of cuticular structures of the pharate adult. While they apparently fulfil similar functions as other storage proteins, tyrostaurins are fundamentally different not only in their smaller but highly variable size (30–65 kDa) but also because they are never released into the hemolymph. Instead, these poorly soluble proteins appear to be deposited into protein granules immediately after being synthesized. No structural or sequence data are available for these interesting proteins, which may represent an alternative mechanism for amino acid storage that has evolved independently. In fact, some coleopteran species possess arylphorin only, but no tyrostaurins, others only tyrostaurins and some express both proteins.

Conclusions

All hexamerins are clearly related to each other and to their proposed evolutionary ancestor hemocyanin, but nothing suggests close relationships between hexamerin families from different insect orders, even if they are similar in their amino acid composition. The structure of the hemocyanin molecule contains many hydrophobic amino acids. Conservative substitution with other hydrophobic amino acids, including aromatic residues, is unlikely to lead to major changes in the overall structure, and therefore an "arylphorin" may have evolved independently in different orders, in response to the need to store aromatic amino acids. This hypothesis is supported by the fact that the position of aromatic amino acid residues are not more conserved in arylphorins than in the other hexamerins; in fact, when all insect hexamerins are aligned with the Clustal W multiple alignment program, phenylalanine and tyrosine residues are common in many positions in all hexamerins (Fig. 1). Not a single aromatic amino acid residue is conserved in all arylphorins but not

in any other hexamerin. Some coleopteran species, on the other hand, may have used a different strategy to store aromatic amino acids, in the form of insoluble tyrostaurins.

Storage proteins other than arylphorins may also have evolved independently in response to the need to store amino acids during the pupal stage, taking advantage of the strongly expressed hemocyanin gene, but other proteins, serving completely different functions, have also evolved from the hemocyanin ancestor. These include the hexamerins from hemimetabolous insects and the above mentioned riboflavin binding hexamerins that are not taken up into fat body. Moreover, other non hexameric insect proteins have been discovered that have a clear sequence homology to hemocyanin: prophenoloxidases, abundant hemolymph proteins, were sequenced from *D. melanogaster* (Fujimoto *et al.*, 1995), *B. mori* (Kawabata *et al.*, 1995) and *M. sexta* (Hall *et al.*, 1995) (sequence homology 29–39%) and a somewhat weaker homology was also found for the arylphorin receptor from Dipteran species (Burmester and Scheller, 1995b; Chung *et al.*, 1995; see below).

PROTEIN STORAGE AND UTILIZATION

Some hexamerins are not taken up and stored by fat body and hence are not storage proteins, while proteins of different evolutionary origin can serve as storage proteins. Essential for a storage protein is its specific recognition and uptake by the fat body. Ever since their discovery it has been questioned why storage proteins are released into the hemolymph after their synthesis in fat body, only to be taken up by fat body just a few days later. Several hypotheses have been offered to explain this seemingly complex pattern, but no uniformly convincing explanation has arisen. It may just be an evolutionary consequence of the availability of ubiquitous, water-soluble hemolymph proteins (hemocyanins). Their storage in fat body required the evolution of an efficient uptake mechanism. Storage protein uptake occurs only during a brief time period shortly before and after pupation, and this uptake is an endocytotic process. Since storage proteins are normally present in large concentrations in hemolymph, non-selective endocytosis alone would assure the import of large amounts of storage proteins into fat body, and initial experiments with horseradish peroxidase demonstrated this (Locke and Collins, 1968). However, the clearing of proteins from hemolymph and the accumulation in fat body is not a function of their original concentration, indicating that the uptake occurs in a selective, receptor-mediated process (Pan and Telfer, 1992). Such a process would not exclude the unspecific import of other abundant hemolymph proteins, since the lumen of endocytotic vesicles would always enclose a small volume of hemolymph. Indeed, when fat body of *H. zea* was incubated with equal amounts of labeled arylphorin and a foreign protein (IgG) *in vitro*, a small amount of IgG accumulated in the tissue, but a tenfold excess of arylphorin was taken up (Wang and Haunerland, 1994b). To achieve the observed selectivity, the uptake must be mediated by specific endocytotic receptors. Potential candidates for such storage protein receptors have been found in both dipteran and lepidopteran species.

Dipteran receptors

Evidence for an arylphorin receptor in dipteran fat body dates back to 1983, when Ueno *et al.* (1983) reported that fat body membrane fractions of the fleshfly, *Sarcophaga peregrina*, bind radiolabeled arylphorin in a specific and saturable manner, with a $K_d=4\times10^{-9}$ M. Various sugars did not seem to influence binding, in contrast to most known endocytotic processes in other animals. The binding of arylphorin requires Ca^{2+} ions and is pH dependent, occurring optimally at pH 6.5, the pH of the hemolymph. In a subsequent study, these authors found that a flavin, lumichrome, prevented binding of arylphorin to its receptor (Ueno and Natori, 1987). In the presence of Ca^{2+}, lumichrome bound to arylphorin in stoichiometric ratios (1 molecule per subunit), suggesting that the lumichrome-arylphorin complex could no longer be bound by the receptor. The authors proposed that a lumichrome-like molecule may be part of the arylphorin binding site of the receptor, and that this molecule can participate in the recognition of arylphorin. With ligand blotting techniques it was demonstrated that the putative arylphorin receptor has a molecular weight of 120 000 Da. Binding of arylphorin was detectable only in pupal fat body, but not in larval fat body, unless the insects had been treated with ecdysteroids. Other evidence indicated that the receptor is present in larvae as an inactive precursor (M_r=125 kDa), and that this "cryptic receptor" is proteolytically cleaved to give rise to the active 120 kDa protein (Ueno and Natori, 1984). Chung *et al.* (1995) have recently cloned the putative arylphorin receptor and have re-assessed their earlier conclusions. It now appears that the 125 kDa protein, previously called the cryptic receptor, is an unrelated protein. Moreover, since it was shown that the fat body membrane preparations contained numerous protein granules, it is now questioned whether the 120 kDa receptor is actually a membrane protein, or instead an intracellular protein that binds arylphorin within the granule. The latter possibility was favored by the authors since they failed to detect the 120 kDa protein, or crossreacting material, with affinity-purified antibodies in fat body cell membranes, but saw strong fluorescence in protein granules. Interestingly, immunoreactive proteins were detected in both larval and pupal fat body, although only the latter can bind arylphorin. In western blots it was demonstrated that both fat bodies contain three immunoreactive proteins, of 120, 76 and 53 kDa, but that the larval tissue has only traces of the 120 kDa protein, which was shown to bind arylphorin. Amino terminal sequence analysis provided conclusive evidence that the smaller proteins are fragments of the 120 kDa protein. Therefore, it was assumed that in larval fat body the 120 kDa arylphorin binding protein

is rapidly cleaved into the two fragments, possibly by a specific protease. The presence of 20-hydroxyecdysone before pupation may inactivate the protease, so that the intact arylphorin binding protein prevails. However, 20-hydroxyecdysone also induces the expression of the 120 kDa protein itself.

Its cDNA, which codes for 1163 amino acids, starts with an endoplasmic reticulum-targeting sequence, followed by the N-terminal sequence of the 120 kDa protein. This is also the amino-terminus of the 76 kDa fragment, which is 695 residues long. The 53 kDa fragment starts at amino acid 713. The sequence shows clear similarity (29.1% identity, 66.2% homology) to a previously sequenced protein from *Drosophila* fat body with unknown function (*fbp1* protein, Maschat *et al.*, 1990), and to the independently cloned and characterized arylphorin receptor from *Calliphora* (Burmester and Scheller, 1995a; 46.1% identity) (Fig. 2).

In *Calliphora* fat body membranes, three arylphorin binding proteins had been found in 1992 by Scheller and co-workers (Burmester and Scheller, 1992), with molecular weights of 130, 96 and 65 kDa. In a direct ligand blot with radioiodinated arylphorin only the 96 kDa protein was labeled, but all three proteins became visible when blotted proteins were first incubated with the ligand arylphorin and then with anti-arylphorin antibodies, a more sensitive procedure (combined ligand immunoblotting). *In vitro* translated mRNA however produced only the 130 kDa protein. All three proteins cross-reacted with specific antibodies raised against each protein and therefore the 96 and 65 kDa proteins appear to be fragments of the 130 kDa protein. Both the complete 130 kDa protein and the 96 kDa protein were prominent in larval fat body before arylphorin uptake takes place; the 65 kDa fragment is absent earlier in larval life, but rises with the initiation of arylphorin uptake. Since both the uptake of arylphorin and the appearance of the 65 kDa arylphorin-binding protein (as well as another 30 kDa protein) is induced by 20-hydroxyecdysone, it was concluded that the 96 kDa protein must be modified before arylphorin uptake can take place, possibly by cleavage to the 65 kDa protein, which may be the active arylphorin receptor (Burmester and Scheller, 1995a). The 96 kDa protein itself is a fragment of the initial 130 kDa gene product. In addition to a clear sequence homology with the *Sarcophaga* receptor and the *Drosophila fbp1* gene (27.8% identity), the receptor is also related to its ligand arylphorin (17.8% identity).

As mentioned above, the sequences of the arylphorin binding proteins from *Sarcophaga* and *Calliphora* are very similar and, hence, we must assume that these are the equivalent proteins in the two species, although their reported function and activation appear, at a first glance, contradictory. However, when considering the aligned

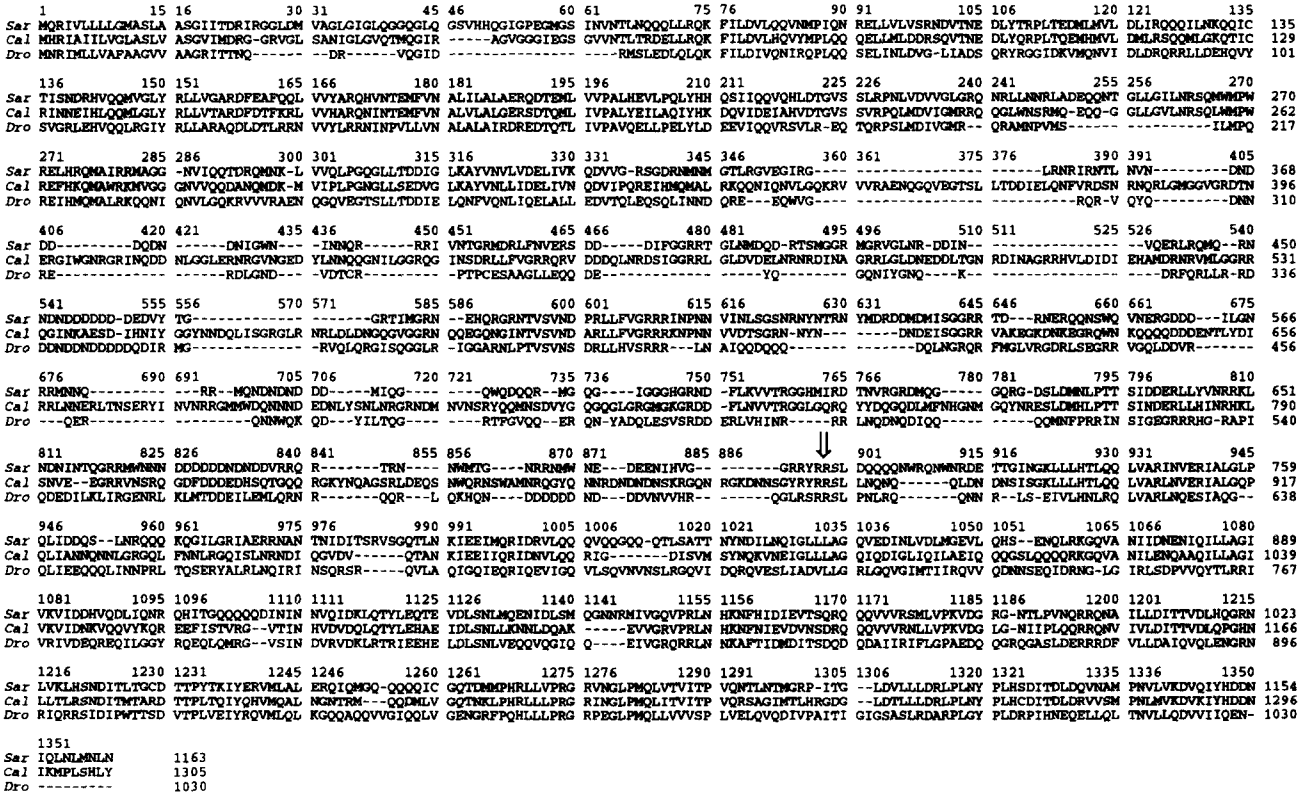

FIGURE 2. Multiple sequence alignment of putative arylphorin receptors from Diptera. Sequences were aligned using the ClustalW algorithm. The arrow indicates the proteolytic cleavage site found in *Sarcophaga bullata*. Sequences were downloaded from Genbank files: Arylphorin receptor from *Calliphora vicina* (Genbank sequence ID 630903); storage protein binding protein from *Sarcophaga bullata* (Genbank sequence ID 984655); fat body P1 protein from *Drosophila melanogaster* (Genbank sequence ID 544281).

sequences, the results from both studies become more congruent. The sequence of both cDNAs contain an endoplasmic reticulum-targeting sequence at their 5′-end. Burmester and Scheller (1995b) postulate a single membrane spanning alpha-helix (I 936–L953); in the aligned sequences of the *Calliphora* and *Sarcophaga* receptors (Fig. 2), this region is very well conserved (61% identity, 89% similarity) indicating that the *Sarcophaga* protein could also reside in the plasma membrane. The total protein is 90 amino acid residues longer in *Calliphora* and the calculated molecular weights correspond fairly well with the sizes found in SDS electrophoresis. (Table 2). The cleavage site was determined for *Sarcophaga* only; it occurs between R713 and S714. In the aligned sequences, *Calliphora* has an analogous sequence at this site (R824/S825), with a conserved stretch of 6 amino acids (RYRRSL) present in both sequences (Fig. 2). This could indeed represent a unique cleavage site for a specific protease. If cleavage occurred here in *Calliphora* as well, the resulting fragments would have a molecular weight of 92 000 and 48 400, respectively (Table 1) and be equivalent to the 76 and 53 kDa proteins found in *Sarcophaga*. In both species, the receptor is inactive unless 20-hydroxyecdysone is present. Different in the interpretation of the uptake events is that Chung *et al.* (1995) suggested that the cleavage of the 120 kDa protein is prevented by 20-hydroxyecdysone, keeping the active arylphorin binding protein intact, while Burmester and Scheller provided evidence that the 96 kDa protein is activated by 20-hydroxyecdysone, probably through further cleavage to the active 65 kDa receptor. However, it should be noted that the postulated membrane-spanning helix would be located in the smaller fragment (840–859 or 937–953), if cleavage occurs in both species at the same position, which would not be consistent with a receptor function of the 96 kDa protein or its 65 kDa fragment. Moreover, the smaller fragment is much more conserved than the larger one (Table 2). Since it is rather unlikely that clearly homologous proteins are subject to fundamentally different regulation in two closely related dipteran species, it appears that different experimental methodologies used in the two studies may be responsible for the contradictory results. While convincing evidence is presented by these and other authors that 20-hydroxyecdysone is regulating the uptake of arylphorin in Diptera, it remains unclear how this is actually accomplished, and whether the arylphorin binding proteins cloned represent membrane spanning endocytotic receptors. Future work will undoubtedly address these important questions.

Lepidopteran receptor

To date, storage protein receptors have been studied only in one lepidopteran species, the corn earworm, *Helicoverpa zea* (Wang and Haunerland, 1993, 1994a). The characterization of the receptor and its localization was directly linked to the two different fat body types observed in this species (see above). Early observations had shown that arylphorin as well as the nonhexameric storage protein VHDL accumulate only in perivisceral (pupal) but not in peripheral (larval) fat body, indicating that specific uptake takes place only in the former tissue (Haunerland *et al.*, 1990). To investigate the possibility of a receptor for these storage proteins, VHDL and arylphorin were radioiodinated and incubated with a fat body cell suspension. Both proteins interacted with fat body cells in a specific, saturable manner; moreover, the binding constants calculated were similar, 7.8 10^{-8} for VHDL and 9.2×10^{-8} for arylphorin (Wang and Haunerland, 1993, 1994a). Bovine serum albumin, in contrast, did not interact with the membranes and had no influence on the binding of either VHDL or arylphorin. When membranes were incubated with labeled arylphorin in the presence of an excess of unlabeled arylphorin, less radiolabel was bound by the membrane; similarly, cold VHDL reduced the binding of labeled VHDL, as expected for a saturable receptor. In addition, unlabeled arylphorin also reduced the binding of labeled VHDL and unlabeled VHDL reduced arylphorin binding, suggesting that a single receptor mediates the uptake of these two structurally different storage proteins. The binding to both proteins occurs between pH 6.5 and 7.5 and requires at least 4 mM Ca^{2+}, similar to the binding data observed in *Calliphora*, but the dipteran proteins appear to bind arylphorin more tightly.

In order to purify the receptor protein for VHDL, membrane proteins extracted from perivisceral fat body were separated by SDS gel electrophoresis. When the

TABLE 2. Comparison of storage protein receptor fragments

Sarcophaga peregina		*Calliphora vicina*		
Reported M_r[a]	Calculated M_r[b]	Reported M_r[c]	Calculated M_r[d]	Identity[e]
120 000	132 346	130 000	148 255	46%
76 000	81 964	96 000	99 986	40%
53 000	50 400	not detected	48 443	55%

[a]Reported by Chung *et al.* (1995).
[b]Calculated from the sequence published by Chung *et al.* (1995).
[c]Reported by Burmester and Scheller (1992).
[d]Calculated from the sequenced published by Burmester and Scheller (1995b).
[e]Determined by pairwise alignment with the ALIGN program (Myers and Miller, 1988).

sample had been treated only with mild conditions, the separated membrane proteins retained their affinity to VHDL. After blotting membrane proteins to nitrocellulose, one protein band could be seen at a molecular weight of 80 000 that bound labeled VHDL. This band constituted a major protein band of the membrane extract. This ligand blot was used as an assay during the isolation of the VHDL receptor (Wang and Haunerland, 1993). Through as series of chromatographic steps this receptor protein could be purified to homogeneity. The receptor is a basic, glycosylated protein of 80 kDa with a high isolelectric point (pH 8.2). Binding of both storage proteins in ligand blots was also competitively reduced by excess of either unlabeled protein, but not by albumin, confirming that a single storage protein receptor binds arylphorin and VHDL. While no conclusive evidence exists for a motif that recognizes two apparently different storage proteins, it has been proposed that the high mannose carbohydrate structures of both proteins could be involved, as well as ionic interactions between the highly positively charged receptor and its negatively charged ligands. If indeed one storage protein receptor was generally responsible for the uptake of all storage proteins, then proteins lacking carbohydrates should not be incorporated. However, methionine-rich protein is taken up by fat body, although it is not glycosylated. It is interesting to note that the glycosylated riboflavin-binding hexamerin characterized in several Lepidoptera is not sequestered by fat body; it may be possible that the flavin prevents binding to the receptor, as it was observed in a dipteran species by Ueno and Natori (1987; see above).

Antibodies generated against this receptor were used to analyze its distribution within the insect. The receptor protein could be found only in perivisceral fat body and only during the latter half of the last larval instar. With electron microscopy and immunogold labeling techniques, it was shown that the VHDL receptor mostly lines the fat body plasma membrane, but is also visible within protein granules (Wang and Haunerland, 1992). This observation is consistent with its role as endocytotic receptor. The receptor appears to be present only briefly, but at very high concentrations. Antibodies detected receptor protein in fat body membrane extracts only between day 4 and 8 of last instar larvae (Wang and Haunerland, 1993). During this period the receptor seems to be the most prominent membrane protein, suggesting that, in contrast to other endocytotic receptors, the lepidopteran storage protein receptor is not recycled, but incorporated into the protein granules. Indeed, the presence of the receptor in protein granules supports this notion.

Through a series of *in vivo* and *in vitro* uptake experiments with gold-labeled VHDL and arylphorin the uptake process could be elucidated (Wang and Haunerland, 1994b). The uptake of storage proteins occurs as illustrated in Fig. 3: storage protein receptor, an integral membrane protein, is synthesized towards the end of the last larval instar and positioned in the plasma membrane,

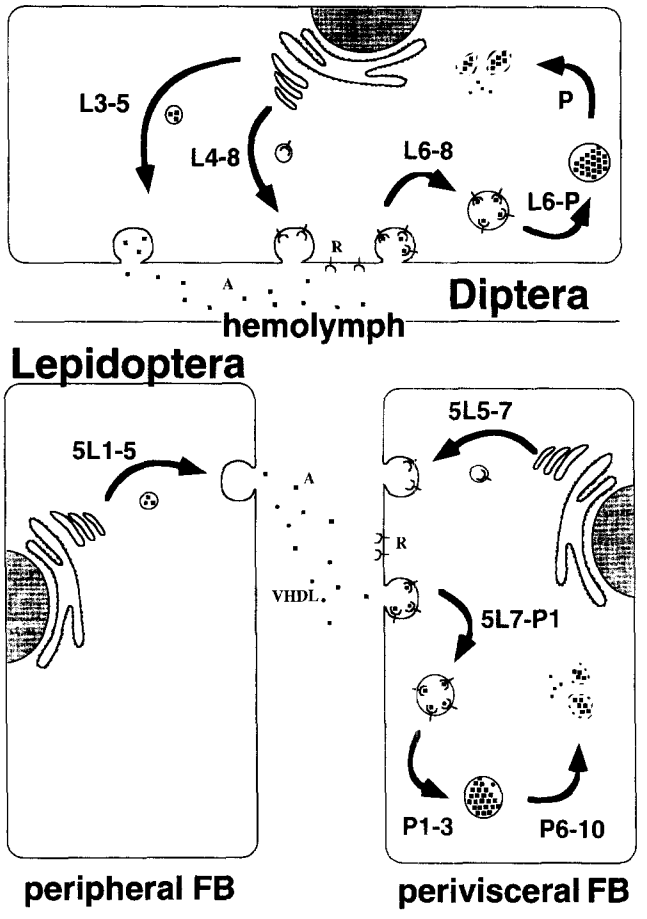

FIGURE 3. Proposed models of storage protein uptake and utilization. Diptera: model for *Calliphora vicina* according to Burmester *et al.* (1995); Lepidoptera: model for *Helicoverpa zea* according to Wang and Haunerland (1994). L3–5, L4–8, L6–8: *Calliphora* larvae 3–5, 4–8, 6–8 days after hatching, respectively. 5L1-5, 5L5-7, 7: 5th instar larvae of *Helicoverpa zea*, 1–5, 5–7, 7 days after the final larval molting; P1-3, P6-10: pupa 1–3, 6–10 days following the larval-pupal molting. A: arylphorin; R: receptor.

with its storage protein binding site facing the hemolymph, and possibly a clathrin binding site facing the cytosol. Once storage proteins have bound adjacent receptors, they aggregate and, possibly through the action of intracellular clathrin, form a coated pit. Eventually the curvature increases until a vesicle containing receptor-bound storage proteins buds off the plasma membrane. Coated vesicles may lose their clathrin coat and fuse with each other to form multivesicular bodies, which contain receptor-bound VHDL and arylphorin, receptors, membrane components, as well as a certain proportion of hemolymph proteins that were enclosed in the vesicle lumen. Soon these multivesicular bodies fuse with primary lysosomes, whose enzymes will digest membrane components and the receptor, so that electron dense protein granules can form. These granules are processed over the next few days, resulting in a gradual digestion of VHDL, leaving behind only arylphorin that can now crystallize and is largely protected from proteolytic digestion. During development of the pharate adult, proteinases partially hydrolyze arylphorin granules, thus

providing the needed amino acids; large amounts of arylphorin remain in partially digested granules until well into the adult stage, when arylphorin may serve as an amino acid reserve for yolk protein production.

Certainly, there are many unanswered questions with regard to the receptor in *H. zea*. What is the common motif that is recognized by the receptor? Will other storage proteins, for example the methionine-rich proteins, bind to the same receptor? Is this receptor common to other lepidopteran species as well? And is there any relationship between the lepidopteran and dipteran storage protein receptors? The latter appears rather unlikely, in view of the different size, processing, control mechanisms and life history, as illustrated in Fig. 3. Considering that storage proteins may have evolved independently after Lepidoptera and Diptera had diverged, it may well be possible that different advantageous uptake mechanisms have also developed independently. Evidence supporting or contradicting this hypothesis, however, can be obtained only after cloning and sequencing of the lepidopteran receptor and the characterization of storage protein receptors in other insect species.

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Note added in proof—Burmester and Scheller (1996) have recently published a detailed phylogenetic analysis of hexamerins and arylphorin receptor. *J. Mol. Evol.* **42,** 713.