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THE FUNCTION AND EVOLUTION OF INSECT STORAGE HEXAMERS

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INTRODUCTION

The storage hexamers are a family of insect proteins with native molecular weights around 500,000 with six homologous subunits weighing generally between 70,000 and 85,000 daltons. They are synthesized and secreted by the fat body of feeding larvae and nymphs and reach extraordinary concentrations in the hemolymph just prior to metamorphosis. In holometabolous insects, they are partially recaptured by the fat body during the larval to pupal molt and stored in cytoplasmic protein granules, as well as in the hemolymph of the pupa. They disappear from these reservoirs during adult development; in many insects, both hemi- and holometabolous, they are also utilized during the nymphal or larval molts. Their amino acids are primarily incorporated into new tissues and proteins during adult development, but they may also be incorporated into cuticle as intact protein, and in one case were found to be diverted to a small degree into energy metabolism. Finally, some storage hexamers have ligand binding and transport capabilities. Functionally, developmentally, and evolutionarily, they are a complex family of proteins whose analysis promises to be exceedingly useful in the study of many basic problems in the biology of insects.

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Scheller (102) edited a timely summary of storage hexamers in 1983. Levenbook (58) reviewed their biochemistry, and Kanost et al (37) treated their molecular biology as a component of a review on hemolymph proteins. The morphogenesis of the secretory and sequestering phases of the fat body is included in a review by Dean et al (14). Hexamers are attracting much attention as model systems for the study of developmentally and hormonally regulated gene function (18, 27, 36, 57, 85, 99, 100), and this popular topic will undoubtedly be reviewed elsewhere in the future. Emphasis is placed in this review on the storage and morphogenetic functions of hexamers and on the evolution that has been driven by these functions.

We introduce here hexamerin as a descriptive and generic term that covers all of the approximately 500-kd hexamers of arthropods. The most prominent members of the family, in addition to the insect storage hexamers, are the hemocyanins that function in oxygen transport in arthropods lacking tracheal systems (61, 126). Less widely recognized is a family of nonrespiratory hexamers in the hemolymph of Crustacea (67). Finally, several unusual hexamers with no antigenic similarity to the more widely occurring storage proteins, but with very similar developmental profiles, occur in great abundance in the hemolymph of several Lepidoptera. This proliferation suggests the recent adoption for storage purposes of cryptic proteins, presumably hexamerins, that have had a long and separate evolution. The proposed origin of hemocyanin from tyrosinase (21) is the kind of conversion that could explain the origin of novel storage hexamers.

Two other widely used terms for what we call the storage hexamers have had great utility, but as knowledge has accumulated they have become, in one case, too restrictive and, in the other, too general. In the first important summary of these proteins, Roberts & Brock (91) summarized their properties, which at that time were understood almost exclusively from studies on Diptera, and adopted the term, larval serum protein, which seemed highly appropriate for that group. But many subsequent studies on Lepidoptera focussed on pupal serum and fat body; and the term applies even less well to the Dictyoptera and Orthoptera, in which two hexamerins become prominent after dorsal closure in embryos and one remains as a major hemolymph protein of adults. A second term, storage protein (119), recognizes a major function of insect hexamerins, but includes by definition many structurally and evolutionarily unrelated proteins, such as the chromoprotein recently described in *Heliothis zea* (33). These terms also lack the advantage of pointing out the relation between the storage hexamers of insects and the hemocyanins, for which evidence is now accumulating (35, 68, 116, 131).

Hexameric structure has generally been inferred from molecular weight estimates for native and dissociated forms of the proteins in polyacrylamide gel electrophoresis (PAGE). Native gels measure, more literally, the Stoke's

radius of the protein and can yield misleading results if applied to proteins that are not spherical, but this is generally not a problem in the measurement of hexamers. More serious is the problem of hexamer dissociation that occurs at the high pH often used in PAGE. Dissociative electrophoresis (SDS-PAGE) yields estimates of polypeptide length, but can be misleading if carbohydrates give the chain a branched configuration, which slows the migration rate (94), especially at high gel concentrations. Consequently, molecular weights inferred from DNA sequencing can differ markedly from those estimated in SDS-PAGE (131). Nevertheless, these methods have been remarkably useful, and any doubts about hexameric configurations have been dispelled by other methods, including analytical ultracentrifugation (76) and chemical cross-linking (49, 115), and by counting the number of native electromorphs yielded by a pool of two kinds of subunits (23).

CLASSIFICATION AND PHYLOGENETIC OCCURRENCE OF THE STORAGE HEXAMERS

Amino acid compositions have been published for 38 storage hexamers in 24 species from 6 orders of insects. These are listed in Table 1, along with the average compositions of 9 arthropod hemocyanins, three nonrespiratory hexamers of crustaceans, [and the 55 animal polypeptides analyzed by King & Jukes (44)]. Tryptophan and cystine/cysteine are omitted from the table because they are not included in many of the published reports. [Cys, when analyzed in the storage hexamers, has very low values. In these proteins, as in several other large hemolymph proteins of insects, both inter- and intrachain disulfide bridges are highly unusual (116).]

For most proteins, a simple percentage composition of amino acids is no longer an interesting topic. Most proteins conform closely to the average composition described by King & Jukes (44). Active site, sequence, secondary, tertiary, and quarternary structure have taken over as the relevant issues in protein chemistry. Composition remains a vital issue for storage hexamers, however, because it is one of the key selective features that have governed their evolution. This proposition was realized at the time of hexamerin discovery, when Munn et al (76) proposed that a high aromatic amino acid content is related to demands for these constituents during sclerotization of the cuticle. This importance is also reflected in nomenclature; *arylphorin* (115) is a designation for proteins with tyrosine and phenylalanine contents totalling more than 15%, and *methionine-rich* describes those proteins in which this amino acid exceeds 4%—in both cases the values are more than twice the average determined by King & Jukes (44) for typical polypeptides.

Two graphs based on Table 1 demonstrate the continuing importance of composition. Figure 1 *(top)* illustrates the effect of plotting the combined

Table 1 Amino acid composition of hexamerins with principal components one and two appended[a]

SUBCLASS: Order/ protein group / *Genus species*	10 x Mole %																Refs.
k:	asx 1	thr 2	ser 3	glx 4	pro 5	gly 6	ala 7	val 8	met 9	ile 10	leu 11	tyr 12	phe 13	lys 14	his 15	arg 16	
HEMIMETABOLA:																	
Dictyoptera/ arylphorin																	
Blatta orientalis LSP	145	54	32	100	71	57	47	66	0	47	65	141	52	57	2	63	c
B. orientalis LSP	136	44	28	98	67	59	53	60	3	40	69	170	51	46	10	66	23
Blattella germanica SPI	121	34	35	118	58	44	52	79	26	38	62	90	65	81	49	47	53
Hemiptera																	
Triatoma infestans	144	44	42	100	50	63	57	78	2	33	96	72	71	71	24	55	87
Orthoptera																	
Locusta migratoria Ap	122	49	65	144	46	51	68	56	8	24	95	90	61	57	13	49	d
L. migratoria LSP-1	88	45	65	117	49	86	99	74	9	37	84	41	49	55	42	60	3
L. migratoria LHP	102	40	45	103	63	70	85	75	2	56	91	69	56	62	12	71	17
L. migratoria PSP	81	33	45	109	64	53	80	66	25	52	90	83	54	39	20	108	3
HOLOMETABOLA:																	
Diptera/ calliphorin																	
Calliphora erythrocephalia	116	48	43	100	31	54	33	59	45	40	58	114	111	82	33	33	16
Calliphora stygia	120	47	42	101	41	54	30	53	38	41	68	119	109	79	31	28	45
Ceratitus capitata 1	139	46	53	110	39	56	47	52	36	45	88	96	71	60	26	36	75
C. capitata 2	143	42	47	115	37	53	44	48	42	42	86	103	73	62	26	37	75
C. capitata 3	142	40	45	112	36	53	46	55	39	43	71	119	75	57	26	41	75
Drosophila melanogaster	128	52	36	103	32	70	41	47	54	37	69	93	94	69	30	42	e
Lucilia cuprina	119	43	37	106	30	51	33	63	35	38	69	118	111	82	29	34	120
Musca domestica	131	41	37	101	39	53	31	53	35	29	50	141	120	94	16	29	66
Diptera/ second hexamerin																	
Calliphora stygia	140	34	62	108	71	58	46	52	20	39	87	94	62	72	50	6	45
Ceratitus capitata	134	41	42	131	50	50	54	69	19	53	66	87	72	60	36	39	74
Drosophila crucigera	155	36	77	111	50	83	57	63	20	28	48	74	61	50	56	32	e
Drosophila melanogaster	127	39	47	115	54	62	46	83	18	28	59	83	82	61	70	26	e
Drosophila mimica	140	49	58	134	36	69	59	68	21	29	63	71	65	62	38	41	e
Drosophila mulleri	150	37	74	113	47	76	52	65	12	31	62	77	71	56	44	32	e
Hymenoptera/ arylphorin																	
Apis mellifera	134	42	78	93	47	58	44	32	57	51	78	96	69	54	12	53	96
Lepidoptera/ arylphorin																	
Bombyx mori	121	45	40	125	61	40	54	61	27	38	69	89	98	85	13	32	123
Galleria mellonella	125	41	37	120	47	48	51	65	18	57	87	100	65	67	13	56	108

Heliothis zea	107	34	55	108	42	50	56	64	21	46	78	104	90	78	30	37	31
Hyalophora cecropia	111	50	49	111	73	49	44	67	15	44	69	92	87	65	36	39	115
Manduca sexta	128	29	52	107	58	49	53	76	20	32	63	103	88	77	29	35	49
Papilio polyxenes	99	64	48	113	60	48	42	67	9	45	72	113	86	73	22	37	97
Calpodes ethlius	100	49	49	72	58	73	39	77	15	32	70	127	84	98	25	32	81
Lymantria dispar	105	46	65	100	51	95	58	64	14	43	74	83	74	72	27	29	38
Lepidoptera/ high met																	
Bombyx mori	122	61	52	86	41	48[c]	35	86	108	46	75	55	51	70	11	55	123
Galleria mellonella	130	53	53	70	39	49	42	65	84	49	86	54	38	88	32	68	108
Hyalophora cecropia	133	59	49	95	23	54	46	70	70	45	86	51	66	85	14	56	121
H. cecropia	136	55	49	96	19	39	36	75	49	52	106	68	62	100	9	52	121
Manduca sexta	130	74	29	35	23	50	29	89	65	65	112	59	57	80	29	74	95
Papilio polyxenes	158	68	20	61	32	42	21	86	39	66	116	62	54	81	22	72	97
Lepidoptera/ other																	
Hyalophora cecropia	109	46	70	108	35	48	45	71	24	68	88	78	48	56	62	43	116
Plodia interpunctella	160	67	34	48	61	47	37	80	48	69	117	36	41	68	19	68	46
Calpodes ethlius SP1[b]	145	67	63	87	30	47	42	59	50	57	101	53	49	63	25	61	81
C. ethlius SP2[b]	134	61	58	30	44	44	38	79	31	54	100	93	68	78	39	50	81
NON-INSECT:																	
Arthropod/ HbCy																	
Average (9 proteins)	139	54	52	110	51	64	63	66	24	46	78	40	53	50	59	51	11,28,67
Arthropod/ non-respiratory																	
Average (3 proteins)	130	54	82	104	72	60	61	71	19	47	72	45	55	46	32	51	67
King & Jukes/ average aa																	
OAA	108	65	85	100	52	78	78	71	19	40	80	35	42	75	30	44	44
Weight factors, f_{ik}, for principal component i																	
i = 2, 100 x f_{2k}:	11	13	15	-4	8	8	17	11	4	14	20	-75	-37	-16	24	20	
i = 3, 100 x f_{3k}:	-15	-18	19	50	21	20	31	-10	-45	-17	-30	-1	-2	-28	20	-18	

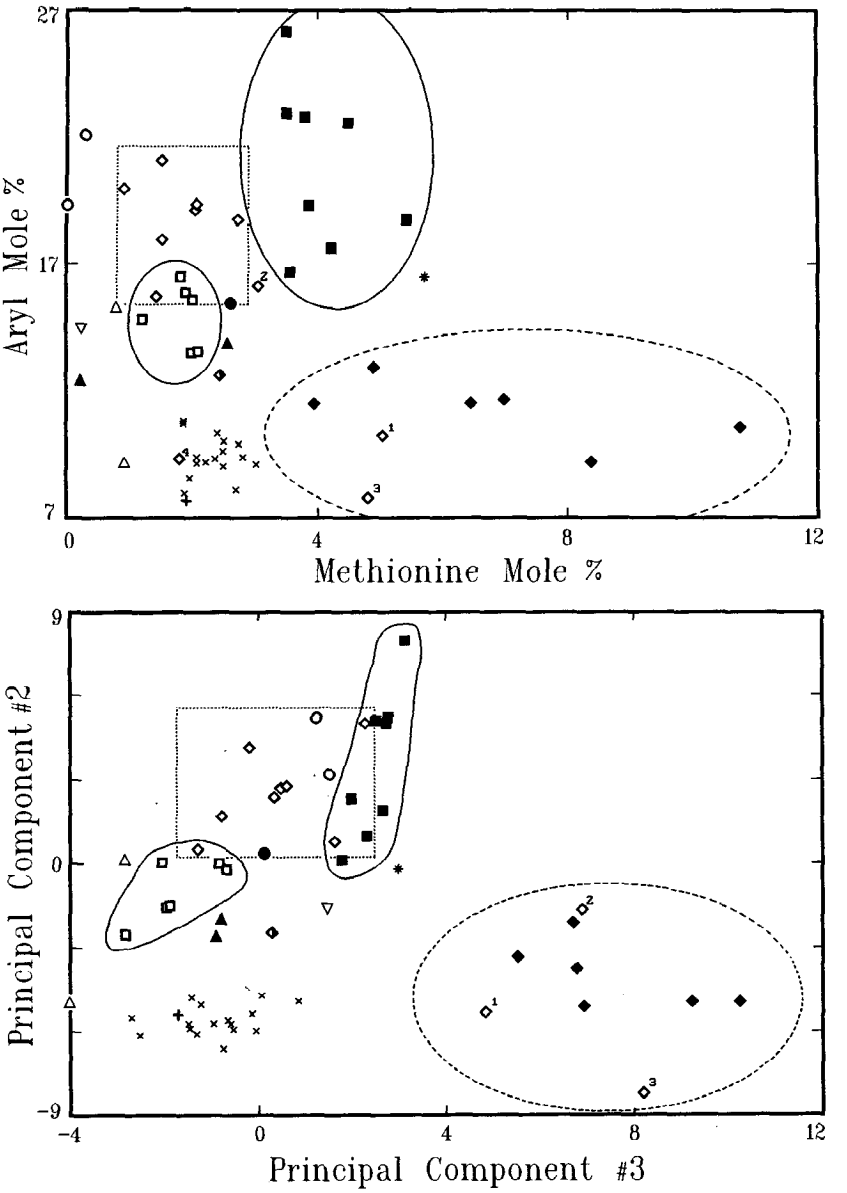
[a]The mole percent was recalculated on the basis of the 16 consistently analyzed amino acids, k, in standard acid hydrolysate analyses. An extra significant digit was allowed in calculations to eliminate rounding errors. Weight factors, f_{ik} (= eigenvectors) for principal components two and three are listed at the bottom of the table. Abbreviations are defined as: LSP, larval serum protein; PSP, persistant storage protein; SP, storage protein; HbCy, hemocyanin; LHP, larval hemolymph protein; OAA, observed average amino acid.

[b]The glycine content of these proteins was very high; the Gly mole percent was adjusted to that of lepidopteran LSP and the mole percents were recalculated.

[c]J. G. Kunkel & R. Duhamel, unpublished data.

[d]D. Philips & B. Loughton, unpublished data.

[e]S. Beverley & A. C. Wilson, unpublished data.



mole-percent of tyrosine and phenylalanine against that of methionine. Each order of insect is represented by a distinct symbol. The symbols cluster in well defined groups and depart markedly from the noninsect hemocyanins and from the King & Jukes polypeptide average. This focus on the aromatics and methionine is not unwarranted because a principal-components analysis of the

compositions in Table 1 (Figure 1, *bottom*) revealed a heavily weighted dependence of the top principal components on the contents of these amino acids.

Phyletic and developmental characteristics tend to parallel the compositional groupings, and we have emphasized this in the organization of Table 1. These sets of properties are summarized in the following text.

The first group (Figure 1, solid squares) is the only one whose aromatic and methionine contents are both high. This dipteran protein was first isolated from extracts of *Calliphora erythrocephala* (77) and given the name calliphorin. It was shown to be synthesized by the fat body (78) and secreted into the hemolymph, beginning about half way through the larval growth period. By the end of larval feeding, it constitutes an extraordinary 60% of the soluble proteins of the insect. In wandering larvae, synthesis stops and much of the protein is endocytosed by the fat body (45) in which it is stored for the duration of metamorphosis.

A protein with very similar composition and developmental profile has been identified and variously named in many species in the suborders Cyclorrapha (8, 16, 39, 41, 45, 65, 73, 77, 132), Brachycera (77), and Nematocera (15). Homology within the Cyclorrapha and Brachycera has been confirmed by antigenic similarities to the original calliphorin. Epitope divergence has occurred for too long a period for this similarity to extend to the Nematocera, and here homology to calliphorin is suggested primarily by biochemistry.

Many Diptera contain a second hexamerin, which has much lower methionine and aromatic amino acid contents than calliphorin (Figure 1, open squares). It occurs in smaller amounts than calliphorin, but follows a very

Figure 1 *(Top)* Mole percent comparisons of the methionine and aromatic amino acid (tyr plus phe) compositions of the arthropod hexamerins shown in Table 1. Each taxonomic category has its own symbol: Diptera *(square)*, Lepidoptera *(diamond)*, Dictyoptera *(circle)*, Orthoptera *(triange pointing up)*, Hemiptera *(triangle pointing down)*, Hymenoptera *(asterisk)*, noninsect arthropods (×), and King & Jukes average (+). Additional distinctions: the persistent storage hexamer of cockroach and locust (solid circle, solid triangles); calliphorin-like hexamers of Diptera *(solid squares)*; high methionine hexamer of Lepidoptera (solid diamonds); footnote b, Table 1 (diamonds 1 and 2). Homologous hexamers are encircled by solid lines (Diptera) and dashed lines (Lepidoptera). *(Bottom)* Principal component analysis of the data in Table 1. To compute the principal component i for protein j, P_{ij}, the loading factors, f_{ik}, are multiplied by their respective moles percent of amino acid k in protein j, and the product summed over all 16 amino acids, aa_{jk}:

$$P_{ij} = \text{Sum } (f_{ik} \cdot aa_{jk}) \text{ for } k = 1,2, \ldots,16.$$

The loading factors f_{2k} and f_{3k} for components 2 and 3 are listed at the bottom of Table 1 below their respective amino acid column. In this figure, P_{2j} is plotted against P_{3j} for each protein. These two components explain the greatest proportion of composition differences. The symbols are identical in top and bottom panels.

similar developmental profile (8, 45, 74, 77, 92). First identified by a unique electrophoretic behavior, it subsequently proved to be antigenically distinct from calliphorin. It has thus far been reported only in the suborder Cyclorrapha. Beverley & Wilson (5), using antisera against this hexamer from five species in the Drosophilidae and one species each in the Tephritidae and Calliphoridae, were able to identify a homologous protein in these seven species, as well as in ten other species of cyclorraphan flies. Only the Phoridae failed to exhibit a cross-reaction, possibly because of early divergence of this family, rather than the absence of a homologue. This protein's synthesis in *Drosophila melanogaster*, in which it is known as LSP2 (91), was recently shown to resume after the eclosion of the adult (107).

The third group (Figure 1, open diamonds) is a lepidopteran glycoprotein with high aromatic amino acid and low methionine contents (31, 38, 49, 50, 81, 97, 115, 123). It was named arylphorin (115) and thus far has been identified in every lepidopteran that has been suitably examined. Its developmental profile is very similar to that of calliphorin; synthesis begins in early or mid-larval stages and terminates in wandering larvae (86, 129), followed by sequestration by the fat body (123). As will be seen below, it seems to be the functional counterpart of calliphorin in lepidopteran cuticle deposition, and the two may well turn out to be interordinal homologues.

The fourth group (Figure 1, solid diamonds) is also a lepidopteran protein. It has high methionine and low aromatic amino acid contents (4, 95, 97, 121, 123), lacks carbohydrates, and also differs from the other three groups in developmental profile. Synthesis by the fat body and secretion into the hemolymph do not begin until close to the end of the last larval instar (86, 129). Then, the fat body avidly resequesters the protein so that its hemolymph concentration in the pupa is often lower than that of arylphorin (95). This hexamerin is the only one known to exhibit sexual dimorphism; in some species, only traces of it occur in males. It does not occur in *Lymantria dispar* (38) or *Spodoptera litura* (122) but otherwise seems to be widely distributed in the order (50). A 70-kd subunit with appropriate amino acid composition occurs in *Plodia interpunctella* (46), but the native protein has not been shown to be a hexamer.

Hexamerins also occur in the hymenopteran *Apis mellifera*, and one of these has been studied in detail (96). Antibodies to *Manduca sexta* arylphorin labelled it in immunoblots of larval hemolymph. This is one of two examples of antigenic similarity between hexamerins in different orders, the other being a monoclonal antibody against tarantula hemocyanin that cross-reacted with calliphorin (68). While the cross-reaction seems to identify the *A. mellifera* protein as an arylphorin, this protein has high methionine as well as tyrosine contents (Table 1), so that its position in Figure 1 (asterisk) is close to the calliphorin cluster. A developmental profile has yet to be published for this interesting protein.

In Dictyoptera (Figure 1, circles), a 16S protein was identified in *Blattella germanica* (53), which proved to be a high aromatic amino acid hexamer that occurs in adults as well as in larvae. A second larval protein, which disappears in the adult, was found in several species of cockroaches, and crossreacts immunologically throughout the order Dictyoptera, including the cockroaches, termites, and praying mantids (51), in confirmation of the taxonomic decision that these groups are closely related. This protein was subsequently purified and shown also to have a high aromatic amino acid content (23). Both proteins initially appear in the embryo at dorsal closure (52, 110). They subsequently cycle in rhythm with molting, reaching a peak concentration a day before each ecdysis, and a minimum shortly after ecdysis (22, 24, 51). The larval-specific hexamer (open circle) gradually disappears after adult ecdysis, while the other (solid circle) persists throughout adult life at a concentration on a par with that of lipophorin.

Locusta migratoria is the only orthopteran in which hexamerins have thus far been described, and here the pattern is much like that of the Dictyoptera (3, 17). The two larval hexamerins have intermediate levels of aromatic amino acids and low or intermediate methionine contents (Figure 1, triangles pointing up). One of these (open triangle) disappears during the adult molt, while the second (solid triangle), whose synthesis is stimulated by juvenile hormone in tandem with that of vitellogenin, persists in the adult (133).

A low methionine hexamer with intermediate aromatic amino acid content (Figure 1, triangles pointing down) was isolated from adult male hemolymph of the hemipteran *Triatoma infestans* taken five days after a blood meal (87). It was not looked for at younger stages, and therefore its role as a storage hexamer is uncertain. We indicate below that it has been implicated in lipid transport.

Several species of Lepidoptera that contain either three or four storage hexamers are exceptions to the general rule of one or two storage hexamers per species. In *Hyalophora cecropia,* fat body extracts and hemolymph yielded two antigenically and electrophoretically distinct high methionine hexamers (121), which are sufficiently similar to suggest that they may have only recently evolved away from each other. Arylphorin was later isolated from pupal hemolymph in this species (115), as was an antigenically and electrophoretically distinct storage hexamer that binds riboflavin (116). In Figure 1, this fourth hexamer (half-filled diamond) falls outside both the arylphorin and methionine-rich clusters. In a phylogenetic search, antibodies to this flavoprotein failed to detect a homologue in other Lepidoptera, including several in the family to which *H. cecropia* belongs (114). It thus appears to have only recently evolved a hemolymph storage function.

Identified storage hexamers of *Trichoplusia ni* include arylphorin and a high methionine homologue (50). The cloned cDNA of a JH-suppressible third hexamer has recently been sequenced and the inferred amino acid

composition found to include only 9.3% aromatic amino acids and 1.8% methionine (35) [Figure 1 *(top)*, diamond 4].

Calpodes ethlius has four storage hexamers (62, 81). One has been identified as an arylphorin (81), and two (including diamond 1) plot in the high-methionine hexamerin cluster in Figure 1. But the fourth (diamond 2) cannot be classified as either an arylphorin or a high methionine hexamer. In *Galleria mellonella* also, arylphorin and a high methionine hexamer have been identified (4, 108), but two other hexamerin subunits (71, 84) have not been characterized. In *S. litura*, antibodies against fat body extracts were used to identify two proteins with developmental profiles in the hemolymph and fat body similar to those of the high methionine hexamer in other Lepidoptera (122). Neither protein crossreacted with antibodies to the high methionine hexamer of *Bombyx mori*. These proteins may therefore be additional examples of the recent evolution of novel hexamerins.

Finally, evidence has been presented for storage hexamers in the hemolymph and fat body of *Diatraea grandiosella* (55) and *Hyphantria cunea* (43). They are not classified here because neither amino acid analyses nor antigenic relationships are as yet available.

DEPOSITION IN THE FAT BODY OF HOLOMETABOLOUS INSECTS

Locke & Collins (63) established the fine structural basis of hemolymph protein uptake by fat body cells by tracing horse radish peroxidase (HRP) injected into last instar larvae of *C. ethlius*. HRP passed from the hemolymph into a reticulum of spaces that contained a concentrate of extracellular materials and lay between fat body cells as well as in narrow channels extending deep into the fat cells' cytoplasm. HRP was also visible in endocytotic vesicles formed at the tips of these channels and, during feeding stages, in multivesicular bodies, which were interpreted as sites where lysosomal enzymes digest it. In pharate pupae, lysosomal activity ceased, and HRP was transferred instead to the newly forming protein storage granules. Over several days of development, the granules continued to accumulate until they occupied much of the cytoplasm of the cells. Similar configurations were described in the fat body cells of pupariating *Calliphora vicina* (70).

Similarities were initially reported between major larval hemolymph proteins and those stored in pupal fat body of *Malacasoma americana* (64), *Pieris brassicae* (12), and *C. erythrocephala* (69). The hexameric nature of the counterparts was established for *H. cecropia* (121), *Bombyx mori* (123), and *C. ethlius* (62). A drop in hemolymph concentration that exactly matches a rise in fat body content implicated endocytosis for both total protein (12, 69)

and specific hexamers (4, 121, 123). Investigators experimentally confirmed endocytosis by injecting labelled hemolymph proteins into larvae and showing that radioactivity is accumulated by the fat body (62, 66, 69, 72, 95, 125). In *M. sexta,* late-fifth-instar fat body sequestered in vitro a quantitative selection of proteins that had been secreted into the medium by fourth-instar fat body (10).

The possibility that storage granules arise from proteins synthesized in situ and immediately deposited without prior secretion into the hemolymph was initially discounted by a decline in the rate of amino acid incorporation by these cells during granule formation (63). In *D. melanogaster,* simultaneous injections of ^{14}C-leucine and ^{3}H-larval hemolymph proteins into late larvae resulted exclusively in ^{3}H labelling of storage granules isolated from the fat body of puparia (118). Hexamer synthesis by the fat body, and more particularly, the capacity for synthesis, implied by the presence of hexamerin gene transcripts, terminates when feeding stops (34, 56, 73, 98, 101, 105, 106, 112, 129). Other evidence, however, indicates a continued low level of synthesis of some hexamerins during the endocytotic prepupal period in some Lepidoptera (72, 86, 129).

In a search for hexamerin receptors, an 800–10,000 G centrifugal fraction of *Sarcophaga peregrina* fat body homogenates bound an iodinated storage protein with a K_D of 4×10^{-9} M (125). Binding was demonstrated in pupal but not larval fat body, unless the latter had been incubated with 20-hydroxyecdysone. The optimum pH and divalent cation dependence of binding were consistent with a receptor that is adapted to operate at the cell surface in the chemical environment of the hemolymph. Finally, evidence was presented that the binding protein is generated during ecdysone treatment from a cryptic receptor with a molecular mass of 125 kd to an active form of about 120 kd (124).

Unambiguous evidence for the selectivity of storage protein deposition in the fat body has only rarely been presented. An intuitive, and probably correct, assumption of selectivity has been based on the overriding predominance of the storage hexamers as constituents of the protein granules, but this hypothesis overlooks the hexamers' corresponding predominance as constituents of the hemolymph at the time of uptake. The process clearly has a nonselective component, as the uptake and deposition of HRP indicates. A peculiar observation from *D. melanogaster* is that fat body cultured in Schneider's medium supplemented with fetal calf serum or bovine serum albumin generates what appear to be storage granules (9, 118). Measurements of the relative contributions of nonselective and selective uptake comparable to those done for yolk deposition (113, 117) have not been reported.

In addition, a puzzling difference separates the fine structural con-

figurations of endocytosis in fat body cells and oocytes, whose endocytosis has been more thoroughly studied. In fat body (63), most of the HRP-labelled endosomes did not exhibit the outer clathrin coat, the inner lining of adsorbed materials, and the clear central lumen characteristic of the vesicles that were found during the 1960s to abound in the cortex of vitellogenic oocytes (2, 93, 109). Only two electron micrographs of single vesicles with apparent clathrin coats have appeared in the literature from *C. ethlius* fat body studies (14, 63), and both of these are homogeneously filled and do not exhibit a clear lumen and a dense lining. Two coated vesicles with the conventional electron transluscent lumen are in fact present in an electron micrograph of *C. vicina* fat body cells during endocytosis (70), but again the frequency is orders of magnitude lower than in the vitellogenic oocyte cortex. A number of studies concerned with the remodelling of these cells during pupation make no mention of the coated vesicles (19, 130). The physiology and fine structure of the fat body's brief endocytotic phase require closer examination to clarify the questions raised by the pioneering studies described in this section.

HEXAMERIN FUNCTIONS IN MOLTING, METAMORPHOSIS, AND REPRODUCTION

Dictyopteran hexamerins and the lepidopteran arylphorins are utilized during larval molting, as well as during adult development (22, 49, 51, 115). In general, larval cycling entails a rise in arylphorin concentrations during feeding and a precipitous drop, in some cases to undetectability, during molting. Synthesis terminates in *M. sexta* at the outset of the molt (86), presumably as a response to ecdysone. Its resumption requires both the withdrawal of ecdysone and the availability of nutrients (128).

In *Blatta orientalis,* the clearing of the two hexamers during the molt results at least in part from a nonselective mechanism that can capture all hemolymph proteins (24). During the intermolt period, the storage hexamers and an equal-sized foreign protein exhibited half-lives of over 100 h, while a small foreign protein, radiolabelled bovine serum albumin, disappeared from the hemolymph with a half-life of only 4.7 h. During molting, by contrast, all three proteins exhibited short half-lives. The results imply that a size barrier separates the hemolymph from a nonselective mechanism of clearance, and that this barrier becomes less restrictive during the molt.

To investigate hexamerin functions during metamorphosis, Levenbook & Bauer (59) injected *C. vicina* larvae that had completed their growth with ^{14}C-phenylalanine–labelled calliphorin. Five days later, when the animals were half way through adult development, the injected label retained the mobility of calliphorin in SDS-PAGE, except for a small amount expired as CO_2. In four-day-old flies, in which calliphorin has disappeared, the label

was widely distributed, with 46.5% in the thoracic muscles, 10.8% in the SDS-insoluble components of the cuticle, and lesser amounts in other tissues. Autoradioagrams of SDS-PAGE gels indicated that large numbers of proteins were now labelled, including especially actin and myosin. Calliphorin had thus served as a source of phenylalanine, and presumably other amino acids as well, in support of the synthesis of adult proteins.

During the first several hours after its injection into seven-day-old larvae, however, radiolabelled calliphorin was found in the cuticle (103). Antibodies to calliphorin stained the endocuticle of larvae, pupae, and adults. In a follow-up of this study (48), intact calliphorin was demonstrated in the cuticle of both third-instar larvae and pupae by immunoblotting of SDS extracts; differential extraction studies indicated that much of the protein may be covalently linked to the cuticular structure. Labelled calliphorin was incorporated into cuticle without degradation, both after injection into the animal and in isolated integuments incubated in Schneider's medium. The plausibility of a role in sclerotization was indicated by the demonstration that calliphorin and arylphorin-type hexamerins isolated from hemolymph can be cross-linked with diquinones in vitro (30).

Undefined cuticle proteins with antigenic similarities to hemolymph proteins had earlier been reported in *Periplaneta americana* (26) and *M. sexta* (47). In *L. migratoria* (83), the antigenic counterparts included the principal larval hemolymph protein, which was later shown to be a storage hexamer (17). In *M. sexta,* arylphorin in particular was shown in immunoblots of SDS-PAGE gels to be extractable from fifth-instar and pupal cuticle (86).

Are these cuticular hexamerins derived from the hemolymph, or are they secretions of the epidermis? In Lepidoptera, translatable arylphorin mRNA was detected in extracts of epidermis (86), and arylphorin itself has been detected in both apical and basal secretions of the epidermis (80). So epidermal secretion is probably the source of at least part of the cuticular form of this protein. In *C. vicina,* by contrast, the fat body appears to be the exclusive site of calliphorin mRNA (105). This observation, in combination with the labelled-protein-uptake studies (48), implicates transport from the hemolymph.

One would anticipate from these results that calliphorin is an indispensible precursor to the construction of the adult, but a genetically based test of calliphorin function devised by Roberts (89) for LSP1, the calliphorin homologue in *Drosophila,* indicated otherwise. Three genes at dispersed loci encode mRNAs for this protein, and combined deletions achieved by Roberts generated flies that completely lacked the ability to synthesize it. LSP1 normally accounts for 9% of the total protein of the larva at the puparial stage, and disappears within three days after adult emergence (92). While the mutants lacking it could complete metamorphosis, feed, mate, reproduce, and

survive stress tests, the fecundity of both males and females was significantly reduced (90). This loss resulted in part from behavioral problems and in part from poor development of the gonads. The recognized metamorphic functions of the protein can apparently be taken over by other proteins, while hitherto unsuspected reproductive effects, which are not manifested until after it has disappeared, cannot.

A function in egg formation was suggested for the high methionine hexamer of Lepidoptera by its greater abundance in females than in males (4, 95, 97, 123). In *Lymantria dispar,* which lacks the high methionine hexamer, arylphorin occurs in metamorphic females in amounts seven times greater than in males (38). While proteins derived from the adult diet are the principal source of amino acids for egg formation in many insects, the lepidopteran mode is to draw on reserves carried over from the larva, and one would expect that this high protein–demanding function would result in sexual differences in the hexamerins. Ogawa & Tojo (79) interpreted the sexual difference in the high methionine hexamerin in *Bombyx mori* in this way. In particular, these authors pointed out that the chorionic proteins of *B. mori* are rich in cysteine (40), and that methionine sulfur is readily transferred metabolically to this amino acid. While this idea seems plausible, methionine is not the primary constituent of the high-methionine protein, and it must therefore serve other needs as well.

A question of paramount importance is whether hemolymph storage protein utilization during the pupal-adult molt is a consequence of general protein turnover, as Duhamel & Kunkel (24) proposed for the last larval molt of *B. orientalis* or of a selective process, such as that sequestering hexamerins in the fat body during the larval-pupal molt. General protein turnover is unquestionably important, for large amounts of foreign proteins injected into *H. cecropia* pupae disappeared from the hemolymph during adult development (82). As in *B. orientalis* nymphs (24), the protein's size was important; 45-kd ovalbumin and 58-kd human serum albumin were cleared substantially more rapidly from male hemolymph than 510-kd vitellogenin, for which the male has no selective uptake mechanism. On the other hand, selective arylphorin and hemolymph flavoprotein utilization was indicated by a more rapid clearance of both of these hexamers, particularly during the last third of the molt.

There is no published information on the site or sites of either selective or nonselective hemolymph protein clearance in Lepidoptera during adult development. However, in adult female roaches, vitellogenin that had been altered by deglycosylation or carbohydrate oxidation lost much of its affinity for the oocyte and was instead cleared primarily by the fat body and the pericardial cells (29). Clearance of foreign proteins by pericardial cells has also been demonstrated in *C. erythrocephala* (13).

A resumption of endocytosis by the fat body, followed at this stage by

lysosomal digestion, rather than by storage, would simply explain selective storage hexamer clearance in the pharate adult. In *C. erythrocephala,* acid phosphatase reaches a peak in the fat body at pupation (127), which can be interpreted as formation of lysosomes for the autophagic digestion of storage granules and cytoplasm that results in the disappearance of this tissue in the adult (14). The pharate adult fat body of Lepidoptera exhibits sexual differences in this regard: in males, storage granules are digested but the cells survive, while females exibit a pronounced reduction of the tissue in association with egg formation but a retention of protein granules in the few cells surviving in the adult (7). Whether a tissue that is so deeply involved in remodelling or cell death is simultaneously the major endocytotic organ of the pharate adult is a question that requires examination.

Some hexamerin functions may not be related to amino acid storage for morphogenesis. Low levels of lipids are often present (reviewed in 58). Calliphorin binds ecdysone with a low affinity, but there is so much of the protein in the hemolymph of larvae that this activity has been proposed to have potential importance as a hormone-clearing mechanism (25). Arylphorin exhibits a relatively nonselective affinity for hydrophobic substances. This property has been proposed to be a mechanism for dealing with xenobiotics (32). And a third example of ligand binding is provided by the riboflavin content of the hemolymph flavoprotein of *H. cecropia* (116).

Finally, the occurrence of a storage hexamer in adult Dictyoptera (53), *L. migratoria* (133), and *D. melanogaster* (107), and of a putative storage hexamer in *T. infestans* (87), suggests other functions. Lipid content in the adult hexamer of the triatomid includes an unusually high proportion of free fatty acids, which suggests a hydrophobic transport function differing from that of lipophorin.

TERMINOLOGY

The nomenclature of the insect storage hexamers is needlessly chaotic. Though simple, useful terms comparable to lipophorin and vitellogenin, which facilitate communication about other hemolymph proteins throughout the Insecta, are not currently possible, the custom of giving obvious homologues different names in every species examined diffuses the impact of what should be a much more highly regarded and widely understood literature. Within each order, uniform names are clearly possible, and the people active in those domains owe it to themselves to agree on a uniform terminology. Arylphorin and high-methionine hexamer serve this function for Lepidoptera, and, for both the Dictyoptera and *L. migratoria*, larval-specific hexamer (51) and persistent storage hexamer (133) prove to be useful designations. Dipteran terminology in particular, however, needs attention.

At the same time, little has emerged from our review to suggest a more global terminology. It is not yet clear whether interordinal terms should be derived from similarities in composition and function or from evolutionary relatedness as revealed by amino acid sequencing. Ideally, the two approaches would lead to consistent suggestions, but this outcome is far from certain. Arylphorin, for instance, which is now beginning to be applied to high aromatic amino acid hexamers in orders other than the Lepidoptera, could well turn out to lump together hexamers of disparate evolutionary history. We have seen that specific hexamers can disappear, as did the high-methionine hexamers in *L. dispar* and *S. litura,* and can emerge anew from unknown corners of the genome, as did the hemolymph flavoprotein of *H. cecropia* and the *T. ni* hexamer recently sequenced by Jones et al (35). Such events, followed by convergent evolution toward common functions, could well result in compositional similarities between proteins of very different origin. As amino acid sequences become available and evolutionary histories are better understood, it may therefore become necessary to choose arbitrarily between composition and evolution as the basis for an interordinal terminology.

In making this decision, polyclonal antibodies, which have been invaluable tools in demonstrating intraordinal homologies, even between different families, will be of limited use. Cross-reactions between orders are rare, and where they do occur their relevance to the establishment of homology is not clear. Binding sites between monomers are highly conserved across a wide variety of hexamerins (131), for instance, and tarantula hemocyanin and calliphorin have recently been shown to share a common epitope (68). Structural relatedness detectable by antibodies can therefore occasionally reflect an ancestry common to the entire hexamerin family.

GENETIC STRUCTURE AND THE EVOLUTION OF THE STORAGE HEXAMERS

Roberts & Brock (91) reviewed the genetics and evolution of the two dipteran hexamers, particularly including the evidence for duplications leading to multigene families. The first indication of such a family was the complex pattern of calliphorin electromorphs in *Lucilia cuprina* (120). The genetics of the electromorphs suggested 12 closely linked genes. A similar family has been demonstrated in *C. vicina* (104), from which 11 members of a larger family (> 20) of closely linked calliphorin genes were isolated as cloned DNA segments. Fragments of three distinct *Sarcophaga bullata* genes were cloned from a genomic library and shown to be related to the mRNA expressed as LSP1 (111). Despite these indications of large multigene organization of the calliphorin gene, its homologues in *D. melanogaster* are limited

to only three genes, corresponding to the alpha, beta, and gamma subunits of this protein (88, 91). And in contrast to the tight clustering of the *L. cuprina* and *C. vicina* gene families, the three *D. melanogaster* genes are located on different chromosomes, which suggests that transposable elements may figure in their duplication (91). A tandem duplication has apparently figured in the evolution of arylphorin, whose alpha and beta subunit genes in *M. sexta* are separated by 7.1 kilobases and have diverged to the extent that their amino acid sequences are now only 68% identical (131). A similar story will presumably emerge in other cases where electrophoretically different subunits are included in a single kind of hexamerin (23).

The second storage hexamer of *D. melanogaster,* LSP2, is represented by a single copy in the genome (1). Here, and in many other insect hexamerins, single subunit types are detected in SDS-PAGE, in contrast to the electrophoretic complexity seen in the multigene family products listed above. Duplications and multigene families may therefore prove not to be general features of hexamerin gene organization.

Doolittle (20) suggested that gene duplication and subsequent divergence account for the origin of most of the great multiplicity of contemporary proteins. Convincing evidence of the origin of new hexamerins in this way is seen not only in the amino acid sequence similarities discussed below, but also in exon/intron structure: the arylphorin genes of *B. mori* and *M. sexta,* and the high methionine hexamer gene of *B. mori,* all include five exons of approximately the same size (27, 100, 131). The intron structure, as might be expected, is less conservative, and shows the wide sequence and length divergence that are consistent with a more neutral role.

The fifth exon of *B. mori* high methionine hexamer is 80 bases longer than that of the arylphorin gene, and this DNA is a major source of the additional amino acids in the resultant peptide (99). The extension is particularly rich in methionine (27%), and thus of particular relevance to the unique composition of this hexamer (79).

Willott et al (131) compared the structural regions of the few insect hexamers and arthropod hemocyanin genes that have at this time been completely sequenced using alignment procedures and genetic distance matrices that can be used to construct phylogenetic trees. Such an approach provides a novel view of hexamerin and arthropod evolution. The resultant tree suggests that the two subunits of *Manduca* arylphorin diverged approximately 100 my BP, based on an assumed figure for the rate of nonsynonomous codon evolution (60). Comparison of the *B. mori* and *M. sexta* arylphorin subunits would place the divergence of the species at 140 my BP, or just before the alpha-beta divergence in the *M. sexta* line of descent. The divergence of arylphorin and high-methionine protein of *B. mori,* based on the same rate of nonsynonomous base substitution, was calculated to have occurred much

deeper in evolutionary time, at 425 my BP, which would presumably preceed even the origin of the Insecta. Some caution is of course warranted in the interpretation of these interesting figures, for they are based on the average substitution rate calculated for 42 mammalian proteins. If hexamerin substitution rates have been faster than this average, the divergence times would be correspondingly more recent.

In another approach to evolutionary timing, Beverley & Wilson (5, 6) found that microcomplement fixation comparisons of the second dipteran storage hexamer in Hawaiian Drosophilidae could be used to fix the branch points in their diversification, in relation to mainland Diptera.

Percentage amino acid identities after sequence alignment of the insect hexamerins with arthropod hemocyanins range between 22 and 28% and are thus not substantially less than the 30–33% similarities between arylphorins and high-methionine protein (131). The unique storage hexamer of *T. ni* exhibited sequence identities of 28% with *M. sexta* arylphorin, 26% with *B. mori* high-methionine protein, and an almost comparable 22% with a hemocyanin amino acid sequence. Much of this sequence conservation is located in regions that have been shown in hemocyanin to form contacts between subunits and to lie near the beginnings and ends of alpha-helical segments. In all three of the sequenced storage hexamers, only one of the six copper-binding histidines is conserved.

The divergence of the hemocyanin from nonrespiratory serum proteins that have very similar size and composition may have occurred as early as 600 my BP, a currently agreed upon minimal age of the arthropods. Nonrespiratory serum proteins are known from several crustaceans and arachnids (67), and the insect hexamerins may be related more directly to these than to the hemocyanins.

The next ten years should bring much progress toward understanding the evolutionary relationships of the hexamerins. Of key importance will be deciding whether a single momentous gene duplication, deep in evolutionary time, gave rise to the paired storage hexamers of Diptera, Lepidoptera, Dictyoptera, Orthoptera, and possibly other orders whose hexamers have not yet been sufficiently examined. Or will such strict interordinal homologies fail to emerge because of evolutionary loss and appearance of new storage hexamers (processes that have clearly been active relatively recently in the Lepidoptera)?

Equally important to studies of hexamerin evolution and homologies will be a better understanding of the selective forces that have led to such great compositional diversity without disturbing the basic hexameric pattern. From the functional considerations described in this review, we may speculate that hexamer conservation has been important at least in part because a molecular size of about 500 kd is important. On the one hand, it allows the hexamerins

to escape the mechanisms responsible for the rapid turnover of smaller proteins and allows the storage of large amounts of amino acids with minimal osmotic consequences. On the other hand, a size of approximately 500 kd may yield the maximum Stoke's radius, permiting diffusion through the basement membranes surrounding the fat body, epidermis, and whatever other tissues are responsible for selective hexamerin removal from the hemolymph during morphogenesis. Compositional diversity and quarternary structure conservation raise fundamental questions about insect physiology and development, and answering these will provide the biological context for understanding the evolutionary history of the hexamerins.

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