4 Yolk Proteins

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1THE STUDY OF YOLK PROTEINS

1.1 Central issues

Knowledge of the chemistry and biology of yolk proteins has expanded explosively in the past decade and promises to be equally as volatile in the present one. Some old issues have been partially resolved but new issues are arising with the application of new technology. Reviews in the past decade (Engelmann, F., 1979; Gong, H. and Chai, C.-H., 1979; Hagedorn, H. and Kunkel, J., 1979) have considered the biology and biochemistry of the principle yolk protein precursor vitellogenin (Vg) as well as those other serum proteins (Wyatt, G. and Pan, M., 1978) likely to be the major or at least minor "contaminants" of any yolk extract. The roles of some of these minor components will be important issues for research in the coming decade.

Data on yolk protein chemistry have expanded considerably now that new techniques of DNA analysis are providing cloned cDNA and genomic-DNA with which to study the relationships between the Vg gene and the final secretion products (Bownes, M., 1979; see Berger, E. and Ireland, R., vol. 2). Our understanding of the genes for yolk proteins in a few select species may soon transcend that encompassing the physiology and biology of the translation products. The primary translation product, or previtellogenin, and the post-translational processing of this protein have also been lively issues. Vg is a particularly good model of a complex protein in which to study processing because of the massive amounts of Vg that are produced (Tata, J. and Smith, D., 1979) and the extensive changes in subunit structure that occur in its physiological lifespan. The primary translation product's size is currently an important issue because it is expected to be a principal conserved property by which the degree of homology of the various egg-bound forms (vitellins) can be determined. The details of post-translational processing are important because they can be investigated to elucidate protein function and can be used to determine molecular and physiological homologies between species. These homologies may be important indicators of conserved functions. The issues of size and processing are at present being resolved with three classes of perhaps non-homologous Vgs emerging (see section 1.6; Harnish, D. and White, B., 1982).

The status and importance of yolk protein was thrown into doubt by the finding that *Bombyx mori* eggs and embryos can survive and develop to hatching in a male milieu without the female-specific protein, Vg (Yamashita, O. and Irie, K., 1980). The significance of this finding is somewhat diluted by the fact that this species' Vg, although the most abundant single protein in the egg, makes up only 40% of the soluble proteins in the egg. Other endogenous ovary-specific (Borovsky, D. and Van-Handel, E., 1980) and non-female-specific proteins (Irie, K. and Yamashita, L., 1980) may also serve as yolk protein. Nonetheless, there are perhaps proteins in yolks of insects in general other than the female-specific vitellin (Vt) that may have roles as important, or in light of the observations on *Bombyx,* more important, to play in support of embryonic development. We will review the debate on lipophorin (Lp), one of those proteins described in several insect orders which may exemplify a minor but important yolk component.

Another confusing point which has not as yet been entirely resolved is the tissue site of Vt synthesis. While it is clear that insect Vg is synthesized in fat body in widely different insect groups (Pan, M. *et al.*, 1969), it now appears that dipteran Vg is also synthesized in a number of ovarian cell types (Srdic, Z. *et al.*, 1979; Jowett, T. and Postlethwait, J., 1980; Brennan, M. *et al.*, 1982). This again raises the problem of homology between Vgs of different groups. Distinct fat body and follicle cell products with separate non-nutritive functions have been proposed previously (Anderson, L. and Telfer, W., 1969; Bast, R. and Teller, W., 1976; Kelly, T. and Telfer, W., 1979). Currently it appears that the higher dipteran Vts from the various tissue sources are all homologous. The interesting cell physiological paradox of apparently identical gene products in different tissues being processed differently, for export in one tissue and for storage in another, is also raised. The resolution of this question may contribute to our basic knowledge of how macromolecules evolve and how they are directed to their destinations.

The question of sexual dimorphism of vitellogenic competence in insects is also in a resolution

stage. While it was thought that both male and female egg-laying vertebrates respond equally to estrogen stimulation by secretion of Vg from the liver, insect males were thought incapable of Vg synthesis (see Hagedorn, H., vol. 8). Neither of the foregoing assertions is correct. Male insects of at least four major orders have been experimentally induced to produce Vg (Mundall, E. *et al.*, 1979; Kambysellis, M., 1977; Dhadialla, T. and Wyatt, G., 1981; Chalaye, D., 1979) and male livers of some vertebrate species, e.g. turtles (Ho, S. and Callard, I., 1980), are reluctant to do so following simple estrogen treatment *in vitro.* Focus of research efforts will surely now be on relative rates of Vg synthesis and hormonal and cellular mechanisms that permit the observed sexual dimorphisms. For example the recent study of Nair, K. *et al.* (1981) has eliminated the post-metamorphic polyploidization of the fat body as the sexual dimorphic mechanism in *Locusta.*

Few of the above issues can be totally resolved without a thorough understanding of the chemistry of the molecules concerned. In the present chapter we hope to provide a description of the chemistry of yolk proteins, and interpret that chemistry to enlighten the important issues at hand: sexual dimorphism, complex protein synthesis, secretion, post-translational processing and macromolecular evolution.

1.2 Constitution of yolk

A yolky egg has substantial stored products in its cytoplasm which transcend the needs of the embryo for survival through early cleavage. (Non-yolky eggs, such as that of the sea urchin or *Amphioxus*, pass through early embryology and hatch quickly to begin feeding and developing at an early embryonic age.) It is interesting that the small amount of reserve material in these "non-yolky eggs" is a protein yolk. Protein storage is thus a normal feature of even "non-yolky" eggs. Yolk stored in larger amounts provides raw materials for more extensive embryonic development to occur before a hatchling has to find its own food. This makes yolk an important topic of study in insects because in the essentially cleidoic (i.e. closed system) eggs of most insects the yolk is the only source of nutrient during embryogeny. Even a viviparous species such as the cockroach, *Diploptera punctata*, deposits substantial yolk to allow development to a larva which can start drinking a uterine milk, the nourishment for extended growth in the female's uterus (Ingram, M. *et al.*, 1977). This makes the study of yolk a nutritional, as well as biochemical and developmental, problem. While other stored components of the egg (mitochondria, ribosomes, lipid, glycogen, uric acid, vitamins, conjugated hormones and minerals) are undoubtedly important in the physiology of the embryo, protein and lipid yolk predominate and this chapter concentrates on the yolk proteins. These proteins are stored primarily in yolk granules, large membrane-bound quasi-crystalline storage vesicles which are formed by the accretion of smaller pinocytotic vesicles (Anderson, E., 1970; Roth, T. *et al.*, 1976).

1.3 Location in the egg and embryo

Yolk granules are relatively evenly distributed in the centrolecithal egg of the insect. An outer cortex of the egg is relatively free of large yolk granules. As cleavage of the egg proceeds, the central yolky area does not take part in any cleavage process and comes to lie within a cavity surrounded by the epithelium of the blastoderm (see Sander, K., *et al.*, this volume). The yolk is thus somewhat separate from the tissues to which it will be providing nutriment, and mechanisms must be established by which the yolk is efficiently accessed and utilized by the developing embryo.

1.4 Function of the yolk

By virtue of its central location in the egg the yolk must be called upon by the peripheral embryo to release its nutriment. This process is not well understood. It may require an extra embryonic cell type, the vitellophage, to invade the yolky area (Tanaka, A., 1976) and actively digest the yolk materials, this cell type serving as an intermediary for the embryo to provide yolk-derived nutritive products. Initially, in the first few days of embryogenesis, the major Vt is not used as a nutriment. Utilization begins on day five of the 18-day (29-30°) embryonic cycle of *Blattella germanica* (Storella, J. and Kunkel, J., 1979; Kunkel, J. and Storella, J., 1983). It is at this time that vitellophages can be first demonstrated to take

up Vt into their cytoplasm (Tanaka, A., 1977). This point in time may correspond to the end of the cytoplasmic subdivision phase of embryogenesis and the beginning of the real growth of the embryonic cytoplasm, analogous to tailbud stage in amphibians when yolk utilization begins and new ribosomes begin to be synthesized (Balinsky, B., 1981).

All embryos require essential amino acids, lipids, vitamins, energy sources and minerals for growth. Unlike vertebrates, insects do not require the large amounts of Ca^{2+} and PO_4^{2-} for bone growth and, as might be expected, the vitellogenin molecules of insects have not evolved as carriers of PO_4^{2-} or Ca^{2+} as has vertebrate vitellogenin.

Extending this line of reasoning might prove fruitful. Judging from the diversity of hatchling insect morphologies and compositions, one might expect yolks of different insect orders to be similarly diverse in what they provide for their embryos. A dipteran yolk providing for a thinly cuticled maggot larva will supply different biochemicals than one from a more thickly cuticled orthopteroid. Whether these different needs are reflected specifically via different compositions of the yolk proteins is not yet established but should be considered.

Besides provision of raw material for nutrition, few other functions of the yolk proteins have been established. A major non-female-specific protein that is found as a "contaminant" in yolk protein preparations is lipophorin (Lp). This protein functions as a hemolymph transport carrier for hydrocarbons, cholesterol and diglycerides (Chino, *H. et al.,* 1981a; see Chino, H., vol. 10) in larval and adult males as well as females. It is found in the yolk granules of the egg in higher proportion than other serum proteins, and may play a role in lipid transport within the developing egg. Whether Lp is found outside of yolk granules, and whether it can function in diglyceride release and transport between the fat droplets of the yolk and the developing embryo, are critical questions that need answers. In *B. germanica,* Lp is transported into the egg in low levels (Kunkel, J. and Pan, M., 1976) and declines in concentration in embryonic extracts parallel to the decline of Vg slowly until, half-way through the embryonic period, when the embryo begins *de novo* synthesis of this molecule and other serum proteins (Kunkel, J., unpublished). The availability of this protein may be critical in the early development of the embryo when rapid growth puts demands on the yolk for cholesterol, diglycerides and fatty acids for use in membrane synthesis.

From work on vertebrates (Balinsky, B., 1981) it is known that other maternal contributions are stored in the egg and have specific functions during embryogenesis. DNA polymerase is present in sufficient quantities to carry the embryo through the cleavage that occurs during early embryogenesis. Also, sufficient ribosomes and ribosomal proteins are stored in the frog egg for it to reach tail bud stage. Massive storage of ribosomes also occurs in insect eggs (Gall, J. *et al.,* 1969). These molecules have specific functions in the early embryo and, like Vt and Lp, are stored in the egg by maternal efforts prior to ovulation. Thus yolk encompasses a variety of storage materials which may or may not share common processing. The maternally contributed DNA-polymerase and ribosomes are topologically separated by a membrane from the proteins in the yolk granules. Unless the ribosomes and vitellin eventually find themselves within the same autophagic vacuole, they may never experience the same cellular environment. One of the challenges for those interested in yolk function is to pay attention to the cellular compartments in which each yolk protein finds itself, and to follow any changes which may occur during embryonic development. Only fragmentary evidence is available about what happens to insect yolk proteins during and after uptake into oocytes. A suggestion made by Anderson, E. (1970) that different-sized pinocytotic vesicles are involved in Vg uptake has not matured beyond the initial correlation of the size difference with the onset of vitellogenesis. The involvement of Golgi-ER-lysosome (GERL) in vitellogenic ovaries of *Drosophila melanogaster* has been reported (Witkus, E. *et al.,* 1980). Since these organelles have been associated with various aspects of storage and catabolism, it is critical that these observations be extended. The role of bioelectric potentials in the movement of yolk from nurse cells to 6ocytes has been reported in hemipteran and saturniid ovarioles (Teller, W. *et al.,* 1981; Dittmann, F. *et al.,* 1981). In the cricket, two electrophoretically distinct Vts are found in different proportions along the length of the öocyte (Nicolaro, M.-L. and Bradley, J., 1980) posing an interesting problem of either differential

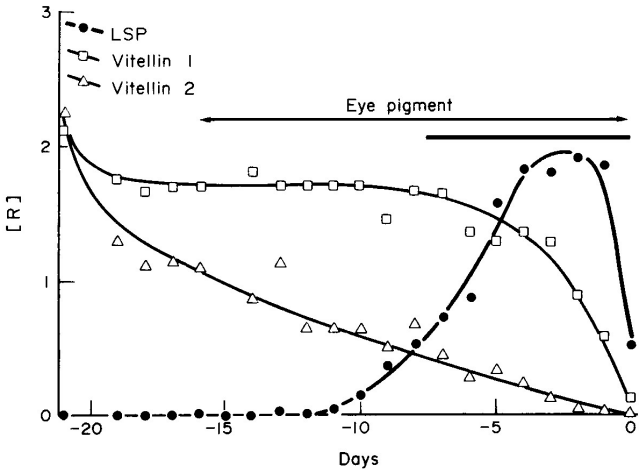

FIG. 1. Differential utilization of two immunologically distinct vitellins during the last 20 days of the 30-day embryonic development of the oriental cockroach, *Blatta orientalis*. Vitellin 1 is utilized primarily during the last 5 days of embryogeny, at a time corresponding to the cuticle deposition phase of the last embryonic molting cycle. The approximate breadth of the last embryonic molting cycle is indicated by the broad horizontal line. The larval-specific protein, LSP, is also first synthesized in the last 10 days of embryogeny. The relative titers, [R], of each protein were measured by quantitative immunoelectrophoresis and are plotted versus days until hatching.

adsorbtion along the oocyte length or sorting out after uptake. This differential distribution of Vts may be the reason why selective utilization of multiple Vts has been observed in some species (Fig. 1), and may reflect differential needs of the late *vs.* early embryo. These observations bear on the more philosophical question of why animals would have multiple genes or types of a storage protein. However we currently know of no differences between Vts within a species which would make multiple types of any particular use to the embryo.

The early studies of W. Teller (1960, 1965) identified major and minor hemolymph protein components of Vt using sensitive immunological techniques. Telfer concluded that only a minority of the proteins was taken up against a concentration gradient. However this conclusion was based on concentration measurements and does not account for possible kinetic factors such as the turnover rate of non-stored proteins. Once a more biochemical approach was adopted, the study of the minor components was abandoned. Only Lp has been studied to any extent beyond relative concentration determinations. It is of great importance for us to establish whether these proteins are synthesized endogenously in the oocyte or are taken up by the oocyte from adjacent or distant sources. It is of considerable interest to know if they are being actively or passively excluded and if their entry in low amounts is via the same vesicular system as Vg.

1.5 Evolutionary homology of yolk proteins

There are strong *a priori* reasons to expect that yolk proteins, and specifically Vts and Vgs might have an evolutionary continuity back to annelids (Thomson, J., 1981). There is, however, evidence which suggests the serious possibility that the Vts of insects do not form a homologous group. The specific issues involved include:

(1) apparent rapid change in the structure of Vt due to its largely nutritive function *vs.* the conservation of certain compositional and structural features;
(2) multiplicity of Vt genes and tissues in which they are expressed;
(3) differences in the primary translation products and their subsequent processing.

Whether the existing information can be best united to support a theory which assumes a single evolutionary origin for the Vt molecules is at the moment debatable. The following information contributes to that debate.

Definitive evidence for homology is hard to establish for the Vts since these proteins lack enzymatic activity and have evolved rapidly (Kunkel, J., *et al.*, 1976; Hagedorn, H. and Kunkel, J., 1979), erasing many traces of similarity. Homology for closely related insects can be established on the basis of immunological cross-reaction. Homology for distantly related species must be argued on the basis of common structural or compositional features. A qualitative indication of cross-reaction can be obtained using Ouchterlony double-diffusion, but a quantitative measure requires greater care. In instances where only one native form of Vt or Vg exists, cross-reaction can be quantified via a precipitin reaction (Praeger, E. and Wilson, A., 1971) or microcomplement fixation, MCF (Champion, A. *et al.*, 1974). However despite assertions to the contrary (Sams, G. *et al.*, 1980), most insects have multiple physical forms of vitellin, for example 14 and 28 S Vt of *Leucophaea maderae*

(Dejmal, R. and Brookes, V., 1972), 18 and 31 S Vt of *Blattella* (Storella, J. and Kunkel, J., 1979) or multiple immunologically or electrophoretically distinct Vgs in *Zootermopsis angusticollis* (Greenberg, S. *et al.*, 1978), *Drosophila* (Bownes, M., 1979), *Acheta domesticus* (Nicolaro, M.-L. and Bradley, J., 1980), *Locusta migratoria* (Chen, T., 1980), *Periplaneta* spp. (Storella, J. and Kunkel, J., 1981) and *Calliphora erythrocephala* (Fourney, R. *et al.*, 1982). Many of these multiple forms seem to be derived from non-allelic loci. These complications make precise quantification of relationships difficult; however, precision may not be important in those instances where large differences in cross-reaction exist. Monospecific antisera can be used to test each immunologically distinct particle's cross-reaction without isolating all the individually compared molecules in pure form from each species. But the different aggregation states of Vt can cause changes in the immunological-equivalence concentrations giving measurable apparent differences where no true sequence difference exists (Kunkel, J., unpublished). One solution to this problem would be to study the proteins, when possible, stabilized in a monomeric state, as has been done with a larval serum protein of Drosophilidae (Beverley, S. and Wilson, A., 1982).

The problem of non-allelic, multiple, distinct Vgs is comparable to the study of hemoglobin (Hb) and myoglobin (Mb) evolution. The multiple globins (i.e. Mb, embryonic Hb, fetal Hb, adult Hb, etc.) not only raise the issue of homology but in addition embellish the problem with degrees or hierarchies of homology which must be resolved before two proteins can be used as reliable indicators of a particular divergence. Differences in a particular Hb subunit for two recently diverged species may be an adequate measure of their length of separation; however, if a Hb subunit of one species had been unwittingly compared to Mb of the other species, one would be examining the more ancient duplication of the globin gene which occurred early in fish evolution. To be accurate, each Vg and Vt must be isolated and studied individually to establish its relationship to the other Vgs and Vts in the animal, and those in related species.

Using MCF, *Blattella germanica* Vg was found to be 100 immunological distance units (IDs) from the Vg of a congeneric species *Blattella* sp. D [near *humbertiana]* (Kunkel, J., Praeger, E. and Wilson, A., unpublished). Experience with other proteins (Champion, A. *et al.*, 1974) suggests that ID is directly proportional to the percentage amino acid sequence difference between two proteins and 100 ID units would correspond to approximately 30-40% sequence difference. This is a huge difference between homologous proteins of congeneric species. However this information is almost useless without a time base on which to evaluate the observed difference. Figure 2 highlights the problem. A relative time base can be established by using other proteins which cross-react throughout the group of interest. This figure shows the ID for various cockroaches relative to the reference using antibodies to *B. germanica* and *Blatta orientalis* larval-specific protein (Kunkel, J. and Lawler, D., 1975) which cross-reacts throughout the order Dictyoptera (including cockroaches, termites and preying mantids). Interspecific MCF differences on the anti-LSP scale are not significantly different

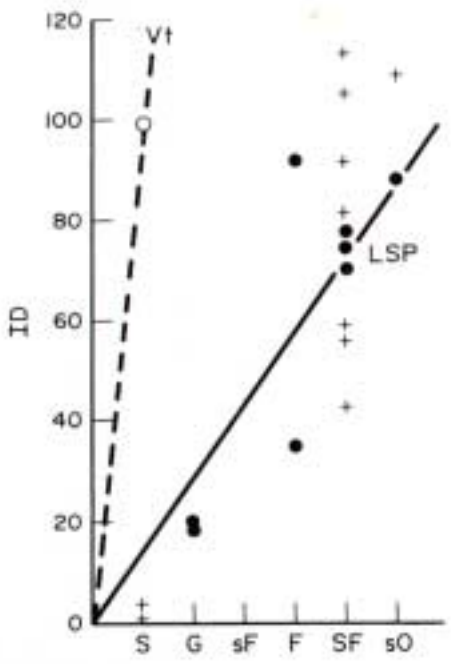

FIG. 2. Relative rates of evolutionary change for vitellin (Vt) and larval-specific protein (LSP). Protein difference is measured as immunological distance (ID), using microcomplement fixation (Champion, A. *et al.*, 1974). A common relative time base is provided by using an ordinal scale of taxonomic distance. IDs using two reference antisera against LSP are plotted separately, (+) anti-*Blatta orientalis* LSP and (•) *anti-Blattella germanica* LSP. Anti-B. *germanica* Vt was used to measure the ID between *B. germanica* Vt and *Blattella* sp. D [near *humbertiana]* Vt (◦) (Kunkel, J. and Lawler, D., 1974; Kunkel, J. *et al.*, 1976; Kunkel, J., Praeger, E. and Wilson, A., unpublished). Taxonomic levels: S, species; G, genus; sF, subfamily; F, family; SF, superfamily; sO, suborder.

from zero. Intergeneric MCF differences using anti-LSP are significant and even allow small spurs of non-identity to be seen using Ouchterlony double-diffusion tests (Kunkel, J. and Lawler, D., 1974). On the other hand Vt at the interspecific level can have changed over 100 ID units, as much as LSP at the subordinal level. Despite these calibrations of relative rates of Vg and LSP evolution, an inadequate fossil record hinders establishing the actual time scale on the taxonomic axis. We are thus not yet able to adequately time events which have occurred during vast spans of evolution within the orders of insects. Accurate statements about relative ages of taxa are at the moment impossible. However, since storage proteins such as LSP occur in both hemimetabolous, as well as holometabolous, orders (Wyatt, G. and Pan, M., 1978; Thomson, J., 1981), they might be choice proteins upon which to base arguments of relative evolutionary rates of the Vts. When combined with plate tectonic estimates of continental separations the immunological distances derived from study of serum storage proteins may give us an accurate account of the relative ages of the diverse taxa.

Using multiple reference points in immunological studies is desirable. The antiserum to LSP of *B. orientalis* views the LSPs of the adjacent *Periplaneta* genus as a single unit with no spurs of non-identity visible in Ouchterlony tests or *B. orientalis* LSP with any of the *Periplaneta* species. This antiserum is in good reciprocal agreement with the placement of both *Blattella* at 106 and *Zootermopsis* at 110 ID units apart from *B. orientalis* using MCF (Fig. 2; Kunkel, J., Praeger, E. and Wilson, A., unpublished) making termites equivalent to another family of cockroaches. With this consistency in mind it is interesting to note that *Periplaneta fulginosa* yolk barely cross-reacts with the four antisera raised against Vt of other *Periplaneta* species (Kunkel, J. *et al.*, 1976).

In contrast, among the Lepidoptera, *Bombyx mori* Vt is reported to cross-react with Vt from a species placed in a separate superfamily, *Philosamia cynthia* (Izumi, S. and Tomino, S., 1980). It is not clear yet:

(1) whether the foregoing variable limits for immunological cross-reaction of Vts correspond to different ages or taxonomic levels within the Dictyoptera *vs.* the Lepidoptera;
(2) whether the Vts of different taxa are diverging at different rates; or
(3) whether the immunology is being confused by the complex nature of Vts.

Either of the two former results would be of non-trivial interest to evolutionary biologists; the later result would make immunology of Vts an unreliable metric of phylogenetic relationships.

The conservation of cross-reaction across broader taxonomic limits is not typical of all Lepidoptera since it has been reported that cross-reaction diminishes to low levels within the speciose genus *Catocala* (Kunkel, J., *et al.*, 1976). Similarly, the restriction of measurable cross-reaction to within seven genera of cockroaches *(Blattella, Symploce, Parcoblatta, Shawella, Supella, Eurycotis* and *Periplaneta)* is not typical of all cockroaches; there is strong cross-reaction of Vts throughout the tropical cockroach subfamily Blaberinae including three genera *(Blaberus, Eublaberus* and *Byrsotria).* This may reflect the recent divergence of the species within the Blaberinae which share an island type ecology (Roth, L. and Willis, E., 1960) consistent with rapid speciation. The yolk proteins could be particularly useful in establishing evolutionary relationships within genera; i.e. the alpha-taxonomic relationships among species (Sneath, P. and Sokal, R., 1973).

1.6 Structural classes of vitellogenins

The only criteria which unify the yolk proteins of different orders are the physical characteristics of composition and size of its subunits. Earlier reports on a variety of insects suggested that all Vgs were composed of large (~100,000 Mr) and small (~50,000 Mr) subunits. However these subunits were soon shown to be products of limited proteolytic cleavage of proproteins of large dimension, ~200,000-260,000 Mr (Koeppe, J. and Offengand, J., 1976; Chen, T. *et al.*, 1978). When the Vt, Vg and the Vg gene of *Drosophila* species were eventually studied it turned out that the later formula did not apply. The three linked genes for *Drosophila melanogaster* Vg (Bownes, M., 1979; Barnett, T. *et al.*, 1980) each codes for a discrete peptide of approximately 50,000 Mr. Other than the processing of a leader sequence (Warren, T. *et al.*, 1979;

Brennan, M. *et al.,* 1980) and addition of oligosaccharide to yolk peptide 2 (Mintzas, A. and Kambysellis, M., 1982), no substantial changes in molecular weight of the peptides occur. With application of sizing techniques to other insect groups a third group of Vgs is suggested (Table 1); besides the large processed precursor molecules and the small unprocessed precursors, there appears to be a group including the mosquitoes and Hymenopterans which has an intermediate Vg precursor length that does not undergo proteolytic processing (Harnish, D. and White, B., 1982). An evolutionary scheme by which all of the current Vts were derived from domains of an ancestral Vg gene product is suggested, but so far only a loose argument supports it (Wyatt, G., 1980) and the distinct possibility of multiple evolutionary origins for Vg must be entertained.

1.7 Genetic approaches to vitellin structure

Both classical and molecular genetic approaches are being used to learn about vitellogenesis (Bownes, M., 1980; Bownes, M. and Nothiger, R., 1981; Postlethwait, J. and Jowett, T., 1980). The finding of a saltatory variation of the size and number of the Vt peptides being expressed in the Drosophilidae (Srdic, Z. *et al.,* 1978) is somewhat confusing despite the better-studied cytogenetic relationships in that group, and this tempers any rush to use Vt cross-reaction as a singularly useful criterion of taxonomic or phylogenetic distance. In *D. melanogaster,* genetic studies have identified three Vt genes: two closely linked (separated by 1.5-2 kilobases) and a third more distant (separated by about 1000 kilobases) from the pair of closely linked Vt genes (Barnett, T. *et al.,* 1980).

This triad of Vt genes may have some evolutionary stability in the higher Diptera since *Calliphora erythrocephala* has also been shown to synthesize at least three distinct vitellin peptides (Fourney, R. *et al.,* 1982). Two of the peptides, which are synthesized in the *Calliphora* fat body, are secreted as a tetrameric native protein of ~200K Daltons. Two peptides are also synthesized in the *Calliphora* ovary. This tissue division of responsibility for Vt synthesis may be a partial reason for the maintenance of multiple genes for what is principally a nutritive function.

Despite this conservation of multiple Vt genes in the higher Diptera, within the Drosophilidae there is substantial variation from species to species as to which of the genes are expressed (Srdic, Z. *et al.,* 1978). Some of these uncertainties may be resolved by closer attention to the physical chemistry of each Vt, the Vg that gave rise to it, and the steps in the synthesis of that Vg. These molecular genetic studies are advancing rapidly.

2 YOLK PROTEIN CHEMISTRY

2.1 Table of yolk protein properties

The physical parameters of enough vitellogenins, vitellins and lipophorins are known such that some interesting generalities can be reached. Table 1 illustrates the molecular weights of the native molecule, the pre-protein and the subunits of the mature proteins. It can be seen, for instance, that the previtellogenins of the class I Vgs are almost all greater than 200,000 M_r. The native Vg is in general a dimer of the preprotein plus added lipid. Only *Manduca sexta* of the class I Vgs departs from this rule. Group II and III do not have sufficient confirmed members to warrant any generalities at this point but a similar rule may apply. Lipophorins have a similarly large native molecular weight.

2.2 Vitellin, the major yolk protein

The major nutritive yolk proteins have been assigned the generic name vitellin (Vt). Precursors of this oocyte protein, found in the bloodstream, have been given the generic name vitellogenin (Vg). However, the differences between Vg and Vt are subtle, when they can be detected at all. In fact Vg and Vt for the majority of insect species appear to be largely identical molecules as evidenced by native molecular weights, amino acid composition and immunological reactivity. In some species endogenous proteolytic cleavage changes the pattern of Vt peptides compared to Vg. Also, subtle differences in their lipid and carbohydrate moieties may exist, although the techniques for measurement of the later parameter have been too imprecise to draw any definite conclusions. We will therefore

Table 1' Chemical properties of vitellogenins (Vg), vitellins (Vt) and lipophorins (Lp). In categories with multiple entries the values are separated with a slash (/). Lipid (Lip) and carbohydrate (Cho)contents are rounded to the nearest percent. The Vt and Vg classes (I III) are as described in Harnish, D. and White, B. (1982)

Source		Molecular Weights (x l0-3)							
				Subunits					
Order		Nat.	Pre-Protein	Large	Small		%	%	
Genus, species	Protein	Mr	Mr	Mr	Mr	Class	Lip	Cho	Refs.
DICTYOPTERA									
Blattella germanica	Vg	652	250p	160/100	50	I	15	7	a
Leucophaea maderae	Vg	559	179/260	118	57	I	7	8	b
Periplaneta americana	Vt	440	-	140/135	62/59	I	-	-	c
ORTHOPTERA									
Locusta migratoria	Vg	550	265/250	120-	-53	I	8	11	d
HEMIPTERA									
Oncopeltus fasciatus	Vt	470	-	160	60/55	I	-	-	c
Rhodnius prolixus	Vt	460	-	160+/160-	59//50	I	-	-	c
LEPIDOPTERA									
Hyalophora cecropia	Vg	516	220	180	47	I	9	1	a,e
Philosamia cynthia	Vg	500	-	120	55	I	-	-	f
Bombyx mori	Vt	440	-	180	42	I	7	3	g
Manduca sexta	Vg	260	-	180	50	I	11	3	h
COLEOPTERA									
Tenebrio molitor	Vt	460	204	160-143	56/45	I	-	-	c,e
DIPTERA									
Drosophila melanogaster	Vt	190	45/46/46	none	44/45/46	II	-	8	c,i
Calliphora erythrocephala	Vt	210		none	51/49/46	II	-	-	j
Lucillia cuprina	Vt	190		none	49/'47/45	II	-	-	j
Aedes aegypti	Vg	350	170+/170-	170	none	III	-	-	e
HYMENOPTERA									
Apis mellifera	Vg	210	190	190	none	III	-	-	c
Blattella germanica	Lp	511	-	none	75--95	-	50	0	a
Periplaneta americana	Lp	600	-	250	85	-	50	1	k
Locusta migratoria	Lp	775	-	200	85	-	36	1	l
Locusta migratoria	Lp	-	85	none	85	-	-	-	m
Manduca sexta	Lp	600	-	285	81	-	37	2	n
Philosamia cynthia	Lp	700	-	-	-		10	1	o

a Kunkel, J. and Pan, M., 1976
b Dejmal, R. and Brookes, V., 1972; Koeppe, J. and Offengand, J., 1976
c Harnish, D. and White, B., 1982
d Chinzei, Y. et al., 1981
e Harnish, D. et al., 1982
f Chino, H. et al., 1976
g lzumi, S. and Tomino, S., 1980
h Mundall, E. and Law, J., 1979
I Warren, T. et al., 1979
j Fourney, R. et al., 1982
k Chino, H. et al., 1981b
l Gellissen, G. and Emmerich, H., 1980
m Gellissen, G. and Wyatt, G., 1981
n Pattnaik, N. et al., 1979
o Chino, H. e[al., 1969
p Wojchowski, D., Kunkel, J. and Nordin, J., unpublished

discuss the chemistry of Vg and Vt together, pointing out differences as the subject arises.

2.2.1 VITELLOGENIN AMINO ACID COMPOSITION

For the majority of large proteins a raw amino acid composition is of little value other than to perhaps confirm a protein's general character, i.e. acidic or basic. Attempts to meaningfully compare amino acid compositions (i.e. Hagedorn, H. and Kunkel, J., 1979) are being supplanted by comparisons of the restriction maps or sequences of the genes. However, Vgs are storage proteins whose precise amino acid sequence may be of little consequence. Based on available immunological evidence, Vts seem to have changed very rapidly during evolution, accepting changes in amino acid composition at a close to neutral rate (Wilson, A. *et al.*, 1977). Their composition, however, remains relatively close to the observed average amino acid, OAA, composition of

a large number of purified and sequenced proteins (Hagedorn, H. and Kunkel, J., 1979) and they provide an interesting example in which composition and structure, but not sequence, are being conserved. The mechanism of this result is not entirely simple. A selective force maintaining the large size of Vgs has been proposed (Hagedorn, H. and Kunkel, J., 1979) based on excretion of small proteins. If the precise amino acid sequence is unimportant, and is being only loosely conserved by evolutionary selection, random mutation could arrive at an average protein composition through the redundancy of the genetic code which biases all mutations towards a predictable average amino acid composition (King, T. and Jukes, J., 1969). Alternatively, real differences in nutritional needs of different animal groups may exert selective forces on the composition of vitellins. For example one might expect the lipovitellogenin of vertebrates to have a higher than average serine content because of nutritional pressure, since this amino acid provides the site for protein phosphorylation. Phosphate and associated calcium, of course, would be needed to support embryonic bone development. Among insect Vts, which seem to all contain the high mannose type of oligosaccharide (see below), one might predict a higher than average asparagine content to provide carbohydrate attachment sites. Both of these latter *a posteriori* predictions are somewhat borne out on examining the Vgs in question. However, these two obvious forces are only components of the nutritional complexity of a Vt.

A number of authors have attempted to compare the amino acid compositions of Vts with each other, and with other serum proteins. The least effective approach is to tabulate the analyses. In some instances attempts to mathematically (Kunkel, J. and, Pan, M., 1976) or graphically (Ogawa, K. and Tojo, S., 1981) compare compositions have led to useful advances in our understanding of these storage proteins. A more general approach to looking at protein composition data reduces the dimensionality from the raw mole percentage values of the 16 commonly analyzed amino acids of each protein to four or five "principal components of variability" (see Reisner, A. and Westwood, N., 1982). One computational approach to obtaining these principal components which is readily implemented on microcomputers is called singular value decomposition (SVD) (Nash, J., 1979). Figure 3 illustrates two components obtained through an SVD analysis of amino acid compositions of insect Vgs and Lps. The first four components account for greater than 80% of the variability in amino acid composition seen in the 21 analyses included. This approach allows one to distinguish between the background noise of analytic differences (obtained within or between laboratories) for a single species' Vg and true differences between species or higher taxonomic groups which may be meaningfully interpreted. The method identifies clear similarities and distinctions between groups of analyses. Overall, the Lps emerge as a distinct class. This is of some interest since a number of groups have suggested that the Vts and Lps have a common evolutionary origin (Mundall, E. and Law, J., 1979; Harry, P. *et al.*, 1979). Lepidopteran vitellins emerge as a separate group, and *Drosophila melanogaster* vitellin is distinct in all perspectives. The *D. melanogaster* distinction is particularly evident if one plots decomposition 1 vs. 3 (not shown) in which all Vts other than *D. melanogaster* plot together, and the Lps are just as far away from the centroid of the Vts as is D. *melanogaster* Vt. In none of the perspectives do the Vts or Lps cluster about the OAA.

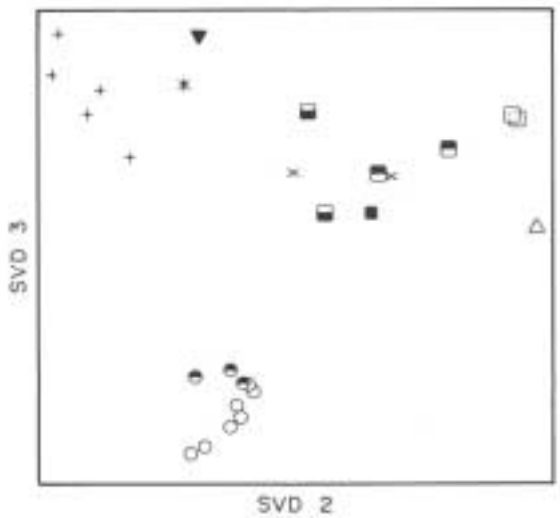

FIG. 3. Two principal components of vitellin (Vt) and lipophorin (Lp) amino acid composition, computed by singular value decomposition, SVD (Nash, J., 1979). The loading factors for SVD 1 and 2 are given in Table 2. Groups of proteins are identified by the shapes of their symbols: lipophorins (pluses), lepidopteran Vt (squares), dictyopteran Vt (circles), locust Vt (crosses), coleopteran Vt (open triangle), *Drosophila* Vt (filled triangle) and observed average amino acid (OAA) composition (asterisk). Identity of each species' symbol can be obtained by referring to Figure 4.

Table 2: *Principal components of variability computed by singular value decomposition of the mole percent amino acid compositions of either lipophorins and vitellins (SVD 1 - 4) or vitellins alone (SVD A D). The percentage of the total variance (%V) explained by that SVD heads each column of loading factors. To obtain a particular SV*D for a *particular protein one sums the products of the loading factor and the mole percent of each amino acid*

	SVD loading factors							
	Vts and Lps				**Vts alone**			
SVD No.:	**1**	**2**	**3**	**4**	**A**	**B**	**C**	**D**
	63%V	6%V	5%V	4%V	64%V	6%V	5%V	4%V
Amino acid								
Asx	43	-27	-45	-20	43	-52	8	-22
Thr	20	-9	2	7	20	-6	9	26
Ser	29	15	-16	13	30	-4	-22	22
Glx	44	61	14	18	46	47	-41	-26
Pro	18	18	3	-31	19	13	17	-23
Gly	19	-22	57	-29	17	31	63	38
Ala	25	8	43	-31	25	39	33	20
Val	26	-29	-4	22	25	-21	2	27
Met	6	17	-19	3	8	-5	-10	16
Ile	18	1	-9	2	18	-5	-2	18
Leu	31	-45	-3	-17	29	-30	30	-19
Tyr	15	25	-15	-18	16	3	-7	-32
Phe	15	-20	-6	20	14	-16	-7	27
Lys	28	7	31	49	27	22	-21	36
His	12	-1	-8	37	12	-7	-27	19
Arg	17	16	-27	-32	18	-13	6	-14

The above approach to amino acid compositional analysis may lead to a deeper understanding of Vt and other storage protein function. Examination of the loading factors for each amino acid (Table 2) which generate each principal component, helps to reveal the underlying forces which are operating. The larger the absolute value of a loading factor, the greater that amino acid's contribution to the principal component. A few comments on the loading factors of Table 2 might clarify the objective:

When Vts alone are included in an SVD analysis a curious phenomenon is observed which is consistent with the storage function of Vts and their probable neutrality to mutations. In a number of decompositions the dominant weighting factors are seen to pair sets of amino acids which are interconvertible by single DNA base changes (i.e. gly-ala and val-leu in SVD B). They have equivalent weights, as if that dimension of variability accepted changes in either amino acid with equanimity. Dimension B when plotted contrasts the cockroach Vts with all the other Vts and thus this phenomenon contributes to a major difference between the Vts. Since the pairing of amino acids in adjacent codon and property groups has been reported as one of the fail-safe and conservative features of the genetic code, it is of some interest that it is able to be seen at such a gross level as amino acid composition in this group of functionally analogous proteins. When other proteins such as the Lps are included in a SVD analysis of Vts the above phenomenon is obliterated, or shifted to other more minor dimensions.

The second through fourth singular-values when plotted against each other reveal differences among the Vts and between the Vts and Lps which have phylogenetic significance. Figure 3 illustrates a plot of SVD 2 vs. SVD 3 which collectively accounts for 12% of the total variability of amino acid composition. In this particular figure the Lps form a distinct group; the cockroach Vts form a group distinct from all other Vts and the locust (Orthoptera), the beetle (Coleoptera) and four moths (Lepidoptera) Vts all cluster as one group. Each SVD accounts for variability but it can only be interpreted as meaningful if it creates a meaningful contrast or cluster when plotted. One can identify particular dimensions with particular groups; thus SVD 2 emphasizes the Lp's difference from Vt; SVD 3 contrasts cockroach Vt with other proteins; SVD

4 and 5 contrast *Locusta* Vt with other proteins; SVD 6 contrasts *Leptinotarsa* Vt to the rest. To a certain extent the significance of a particular dimension is weighted by the number of analyses one has of each Vt type; thus SVD 6 explains only 2.9% of the total variability and its major contrast distinguishes the single sample of *Leptinotarsa* Vg. Some of the more minor SVDs distinguish between individual species of the Lepidoptera. Since we currently have only one dipteran Vt analysis, and it does not cluster with any other group, it is difficult to comment on its relationships to other proteins except to say it is different from all groups studied so far in all perspectives which account for significant variability.

When the Vt composition of the polychaete worm *Nereis bicolor* (Fischer, A. and Schmitz, K., 1981) was included with the insect Vt compositions in an SVD analysis, the worm vitellin was found to be distinct from all the insect Vts and no similarities could be detected other than the superficial ones noted for proteins which resemble the OAA composition.

None of the clusters of proteins center directly about the observed-average-protein composition; however, as remarked earlier (Hagedorn, H. and Kunkel, J., 1979) it is generally appropriate to describe the Lps and Vts as being more similar to the OAA composition rather than to each other. This becomes more obvious when one includes the other "storage proteins" in a SVD analysis. Here in the major dimensions the Lps, Vts and the OAA all cluster closely together, while the "storage proteins" diverge widely from this cluster.

When the amino acid compositions are used to establish a phenetic tree of relationships (Fig. 4) again the Lps are distinct from the Vts, and *Drosophila melanogaster* Vt again is singled out as unique. While the lepidopteran Vts do form a distinct cluster, the depicted relationships of the three families of moths represented do not bear any close correlation to the suggested close immunological ties between the Bombycidae and the Saturniidae. Clearly more amino acid analyses would be useful in determining if the above approaches to probing compositional data of storage type proteins can be of further use. It would be particularly helpful if additional coleopteran and dipteran analyses were available, since the sparse data available now do not allow firm interpretations of their relationships.

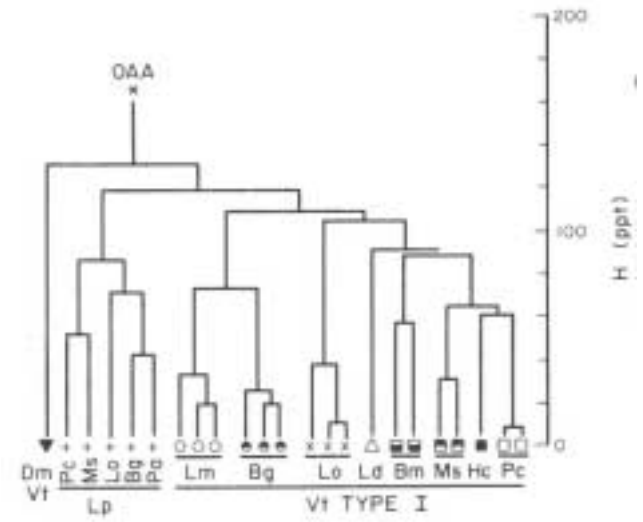

Fig. 4. Phenetic relationships of vitellin (Vt) and lipophorin (Lp) amino acid compositions. The Manhattan distance (It) between all possible pairs of Vt and Lp mole percent amino acid compositions (Hagedorn, H. and Kunkel, J., 1979) were computed and the resulting distance matrix was submitted to a Fitch-Margoliash tree-building algorithm. The choice of branching pattern was guided by clustering indicated by prior analysis of principal components as well as taxonomic considerations. However the best tree was chosen by minimizing a fit statistic and the number of negative branch lengths. One other tree was found with a marginally better fit-statistic. However, that tree had two negative branch lengths versus the above tree's single negative length. The tree was rooted using the observed average amino acid (OAA), composition of King, T. and Jukes, J. (1969). Species abbreviations: **Bg**, *Blattella germanica:* **Bm**, *Bombyx mori;* **Hc**, *Hyalophora cecropia;* **Ld**, *Leptinotarsa decemlineata;* **Lm**, *Leucophaea maderae;* **Lo**, *Locusta migratoria;* **Ms**, *Manduca Sexta;* **Pa**, *Periplaneta americana;* **Pc**, *Philosamia cynthia.* Lepidopterans use square, and dictyopterans circular, symbols. Multiple analyses for many species give a feeling for the errors involved in this technique.

2.2.2 VITELLOGENIN PEPTIDE COMPOSITIONS

Analyses of individual polypeptide chains of Vts of *Manduca sexta* (Mundall, E. and Law, J., 1979), *Drosophila melanogaster* (Mintzas, A. and Kambysellis, M., 1982) and *Leucophaea maderae* (Masler, E. and Offengand, J., 1982) are available. The *M. sexta* polypeptides are different enough from each other that one can use their amino acid compositions to solve a linear equation by which the large and small peptide compositions add up approximately to the total composition of the parent Vt with appropriate equimolar proportions of the subunits. However similar analysis of the three *D. melanogaster* Vt peptides allows no such solution using currently available total Vt analyses.

Clearly more complete analyses of the dipteran Vts are needed. Some of this analysis may be enlightened by gene mapping (Hung, M.-C. *et al.*, 1982) and eventual sequencing which will lead to more precise compositions for each subunit.

Analysis of the more complex assemblage of *Leucophaea* Vt peptides has identified a compositional difference in the peptides cleaved during the transition from 14 S to 28 S (Masler, E. and Offengand, J., 1982). The 9000 Mr peptide cleaved off the D peptide in its transition to the B form is presumably lost since it is unaccounted for in the 28 S form. A predicted composition for the lost fragment is given which suggests that it is high in the amino acids Asx, Glx, Ala and Set, consistent with this peptide being on the surface and suggestive of its containing carbohydrate and/or phosphate.

2.3 VITELLOGENIN OLIGOSACCHARIDE

The carbohydrate constitutions of insect Vgs and Vts published prior to 1977 (Table 1) suggested to us that most if not all of their carbohydrate is present as "high mannose" type of oligosaccharides (Kornfeld, R. and Kornfeld, S., 1976). The salient features of this type of grouping include an invariant diacetylchitobiosyl linkage to Asn and a variable number of mannosyl residues. Figure 5a presents a generalized high mannose structure common to many glycoproteins including ovalbumin (Tai, T. *et al.*, 1977), Chinese hamster ovary cell membrane glycoprotein (Li, E. and Kornfeld, S., 1979), bovine thyroglobulin (Ito, S. *et al.*, 1977) and yeast mannoprotein (Nakajima, T. and Ballou, C., 1974). All high mannose oligosaccharides have a common susceptibility to cleavage by endo-beta N-acetylglucosaminidase H ("endo-H") of *Streptomyces plicatus* (Tarentino, A. and Maley, F., 1974). The minimum high mannose structural feature:

$$\text{Man}(\alpha 1\rightarrow 3)\ \text{Man}(\alpha\rightarrow 6) — \text{Man}(\beta 1\rightarrow 4) —$$
$$\text{GlcNAc-}(\beta 1\rightarrow 4)—\text{GlcNAc—Asn}$$

S required for its activity. This enzyme cleaves the β1→4)glycosidic bond joining the two GlcNAc residues, liberating an oligosaccharide devoid of its reducing GlcNAc residue. Treatment of glycopeptides prepared from delipidated specimens of purified *Blattella germanica* Vt and Vg, and Vts of *Periplaneta americana, S. capitata, Blaberus discoidalis* and *Locusta migratoria* with endo-H showed that their carbohydrate is present as high mannose oligosaccharides (Kunkel, J. *et al.*. 1978, 1981; Nordin, J. and Kunkel, J., 1982).

2-[^{3}H]-Mannose has been used to label the oligosaccharide portion of *B. germanica* Vg. Following SDS-Page essentially all of the radioactivity was found to reside in the large (100K) subunit (Kunkel, J., *et al.*, 1978). Similarly, *M. sexta* and *Bombyx mori* Vt oligosaccharides are attached exclusively to their heavy subunits (Mundall, E. and Law, J., 1979; Susumi, I. *et al.*, 1980). This high degree of conservation of oligosaccharide structure and placement on insect Vgs and Vts suggests a generalized functional role during some phase of the macromolecule's physiological life. Injection of tunicamycin (Tm), a potent inhibitor of protein N-glycosylation (Kuo, S.-C. and Lampen, J., 1974), into vitellogenic *B. germanica* females blocks 2-[^{3}H]mannose incorporation into Vg (Kunkel, J. and Nordin, J., unpublished). In these experiments Vg secretion from the fat body measured immunologically is completely blocked in Tm-treated animals. SDS Page reveals a large (> 200.000 Mr) polypeptide accumulates in the fat body, suggesting nascent peptide is either not processed normally or accumulates because subunit assembly is impaired. A general impairment of fat body secretion by Tm

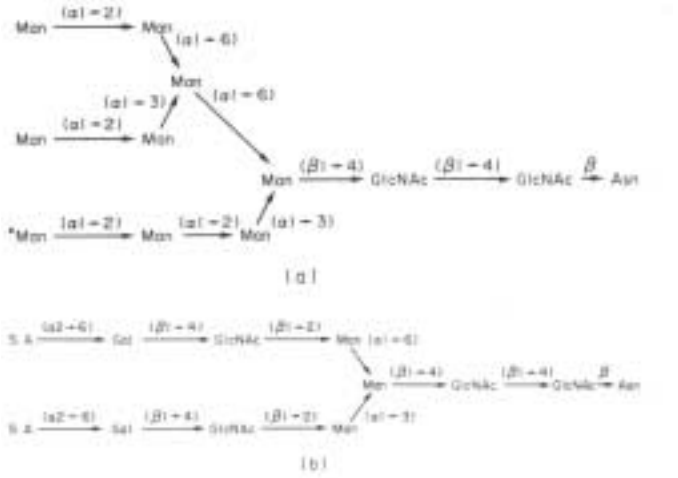

FIG. 5. Asparaginyl-linked oligosaccharides. (a) High mannose type; (b) complex type. Abbreviations: Asn, asparagine; Gal, galactose; Glc, glucose; GlcNAc, N-acetyl glucosamine; S.A., N-acetyl neuraminic acid (sialic acid). The asterisk (*) indicates the attachment point of the trisaccharide [Glc (1-.2) Glc (1-,3) Glc (1~3)] in the dolichyl pyrophosphoryl-linked oligosaccharide.

action has been reported in *Galleria mellonella* (Miller, S. and Silhacek, D., 1982).

Similarly, Tm disrupts the proper assembly of VSV virions because the aglycosyl coat glycoprotein aggregates (Gibson, R. *et al.,* 1979). Merlie, J. and co-workers (1982) report the accumulation of acetylcholine receptor subunits in the presence of this inhibitor. Therefore oligosaccharide chains on Vg, as well as particular locations on the polypeptide, could be a prerequisite for assumption of proper conformation upon assembly. Other possibilities for oligosaccharide function are described below.

2.2.4 MICROHETEROGENEITY OF VITELLIN OLIGOSACCHARIDES

Thin-layer and gel-filtration chromatography of the endo-H oligosaccharides from four cockroach species have revealed that they are not a single size. Although the *B. germanica* and *P. americana* oligosaccharides contain one major and five or six minor components, those from *S. capitata* and B. *discoidalis* contain two major and four or five minor ones (Nordin, J. and Kunkel, J., 1982) (Fig. 6). As observed with thin-layer chromatography (TLC), at least six components can be identified. Control experiments show that the heterogeneity is not the result of contaminating glycosidase activities present during isolation of the Vts or their respective oligosaccharide fractions. Further studies showed that microheterogeneity is characteristic of the oligosaccharide population of newly secreted Vg molecules. Gel filtration chromatography of the *B. germanica* oligosaccharide fraction with [^{14}C]oligosaccharide standard markers established that its major saccharide contains nine mannosyl units.

Oligosaccharides prepared from purified Vt of a single *B. discoidalis* egg case provided a bimodal TLC pattern essentially identical to those purified from pooled Vt of many females (Fig. 6, lanes 3 and 4). Therefore the microheterogeneity and bimodality are properties of a single individual's Vt, and are probably species-specific traits. Some possible causes of the microheterogeneity will be discussed below. *Locusta migratoria* Vt oligosaccharide is also microheterogeneous. Because the locust and cockroach represent separate orders of the class Insecta, it is most likely that microheterogeneity of

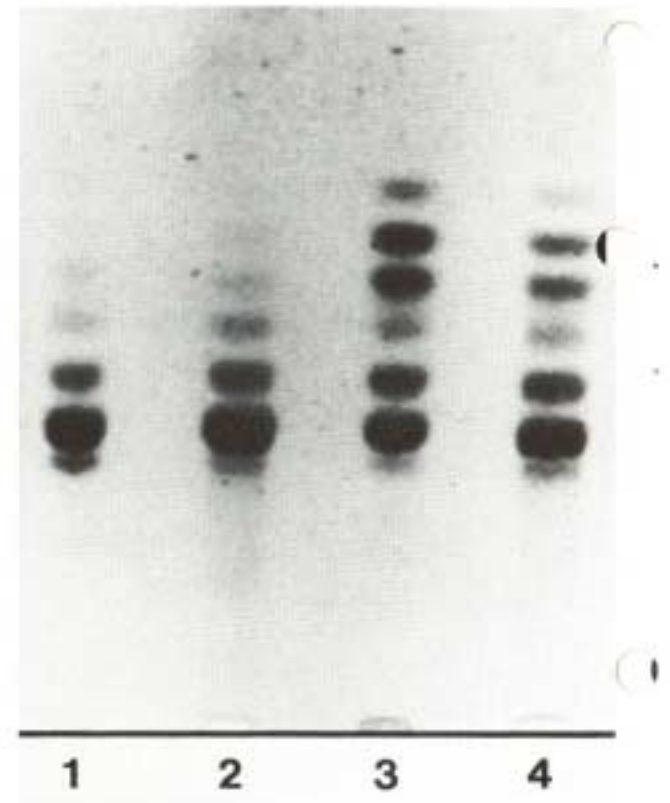

FIG. 6. Heterogenity of vitellin oligosaccharides of three species generated by endo B-N-acetylglucosaminidase H digestion. Separation is by thin-layer chromatography with smallest oligos travelling farthest from the origin. Two types of heterogeneity are evident. Lanes 1 to 3 depict the endo-H oligosaccharides from the vitellins of *Locusta migratoria, Blattella germanica* and *Blaberus discoidalis* respectively. *Locusta* and *Blattella* show a unimodal distribution of oligosaccharide lengths with a mode at Man_6-GlcNAc and microheterogeneity decreasing in both directions. *Blaberus* oligos show a bimodal distribution with modes at Man9 GlcNAc and Man6-GlcNAc. Lane 4 depicts the oligos from a single ootheca of *Blaberus,* showing that such bimodal heterogeneity occurs in the vitellin produced by a single female, as well as in a vitellin batch pooled from multiple females.

insect Vt oligosaccharide is a general occurrence. Oligosac-charide structural heterogeneity of ovalbumin from a single hen has been described recently (Iwase, H. *et al.,* 1981).

Structural and evolutionary relations which exist between the oligosaccharides of insect and vertebrate Vts are now beginning to emerge. Three recent observations bear on this subject. First, of the few insect Vts examined a11 display oligosaccharide heterogeneity (Nordin, J. and Kunkel. J., 1982). Since insect glycans do not contain sialic

Table 3. Lipid compositions of lipophorins, vitellins (Vt) and vitellogenins (Vg). Presented as percentage of total lipid.

Protein		%	Percentage of lipid as				
Genus, species		lipid	DG	CH	PL	HC	References
Lipophorin							
Periplaneta americana		50	15	5	43	28	Chino, H. et al., 1981b
Locusta migratoria		31	42	7	38	nd	Peled, Y. and Tietz, A., 1975
Manduca sexta		37	34	5	38	14	Pattnaik, N. et al., 1979
Philosamia cynthia	H	44	58	14	27	nd	Chino, H. et al., 1969
Philosamia cynthia	E	10	15	6	71	nd	Chino, H. et al., 1969
Vg and Vt							
Locusta migratoria	Vg	9	35	13	51	nd	Chino, H. et al., 1977
Locusta migratoria	Vt	7	5	8	78	nd	Chino, H. et al., 1977
Philosamia cynthia	Vg	7	11	7	75	nd	Chinzei, Y. et al., 1981
Philosamia cynthia	Vt	8	23	8	69	nd	Chinzei, Y. et al., 1981
Bombyx mori	Vt	7	9	6	84	nd	Izumi, S. and Tomino, S., 1980

DG = diglyceride, CH = cholesterol, PL = phospholipid, HC = hydrocarbon, H - hemolymph, E - eggs, nd = not done.

acids (Ng, S. and Dain, J., 1976) it seems improbable that any examples with the complex type chains (Fig. 5b) will be found. Second, early reports (Ansari, A. *et al.*, 1971; Redshaw, M. *et al.*, 1971) described the presence of carbohydrate in amphibian Vt, and more recently Gottlieb, T. and Wallace, R. (1982) showed that the oligosaccharide of *Xenopus laevis* Vt is of the complex type. Third, studies on cultured mosquito embryo cell plasma membrane glycoproteins show that they also contain the high mannose oligosaccharides (Butters, T. and Hughes, R., 1981; Butters, T. *et al.*, 1982). A most unusual aspect of the latter study is the finding that the oligosaccharide is identical in structure to that of the fully synthesized dolichyl pyrophosphoryl oligosaccharide [Glc_3-Man_9-$GlcNAc_2$] (see Fig. 5a and section 3.3), actually the oligosaccharide donor for N-glycosidically linked glycoprotein biosynthesis (Hughes, R. and Butters, T., 1981). This interesting observation indicates this cell line cannot process (Hubbard, S. and Ivatt, R., 1981) the oligosaccharide after its transfer to polypeptide (section 3.6.1). Hughes, R. and Butters, T. (1981) have postulated that there is an evolutionary pattern of oligosaccharide processing capabilities of various organisms in which insects are least, and vertebrates most, able to modify and convert their N-glycosidically linked oligosaccharides to the complex type. This idea is certainly consistent with the observation made with cockroach and locust Vts. Here the predominant oligosaccharide species is Man_9-$GlcNAc_2$. With lesser amounts of Glc_1—Man_9—$GlcNAc_2$ and Man_8—$GlcNAc_2$. Therefore it appears that insect endoplasmic reticulum and Golgi apparatus do not significantly modify these oligosaccharides. The *Blaberus discoidalis* Vt oligosaccharide (Fig. 6) is one notable exception to this rule. Gottlieb, T. and Wallace, R. (1982) have noted that while the oligosaccharide synthetic and processing apparatus of *X. laevis* provide for complex oligosaccharide synthesis, the location of the transglycosylases for synthesis of the oligosaccharide core structure are located in the smooth, not the rough, endoplasmic reticulum as they are in higher vertebrates.

2.2.5 VITELLOGENIN LIPIDS

Lipids are integral to all Vgs and Vts characterized either by lipid-specific staining of polyacrylamide gels (Oie, M., *et al.*, 1975; Gellissen, G., *et al.*, 1976; Atlas, S., *et al.*, 1978; Jensen, P., *et al.*, 1981) or by various quantitative techniques (Izumi, S. and Tomino, S., 1980; Chinzei, Y., *et al.*, 1981; Dejmal, R. and Brookes, V., 1972; Kunkel, J. and Pan, M., 1976; Chino, H., *et al.*, 1977; Mundall, E. and Law, J., 1979). Table 3 summarizes lipid compositional data for four of the best-characterized Vgs.

Considerable similarities exist among the phospholipids, diacylglycerols and cholesterol which comprise the bulk lipid components. While present evidence is too meager to draw any firm conclusions, Vts appear to contain less diacylglycerol than Vgs. The same relationship may also obtain for their total lipid contents. A role of Vg as a carrier of diacylglycerol molecules into the egg for

conversion to triacylglycerol is possible. While conjugated ecdysteroid has been found non-covalently bound to Vt from locust eggs (Lagueux, M. *et al.*, 1981; see Hoffmann, J. and Lagueux, M., this volume), it is not known whether it is transported into the egg attached to Vg. The lower solubility of Vts compared to Vgs could facilitate its aggregation and storage in the oocyte and may reflect loss of some lipid upon uptake from the hemolymph. Utilization of lipid by the developing embryo (Sroka, P. and Barth, R., 1976; Chinzei, Y. *et al.*, 1981; Chino, H. *et al.* 1977) or its mobilization from the yolk cavity to the surrounding embryonic tissue may be aided by its association with Vt.

Nothing is presently known regarding localization of the lipids on the Vg and Vt molecules, although one proteolysis study suggests that the large subunits and associated lipids surround the small subunits (Mundall, E. and Law, J., 1979). Another unexplored aspect of the lipid-protein relationship in Vt is the possibility that some covalent bonding between the two may occur as it does in proteolipids (Schlesinger, M., 1981).

2.2.6 VITELLIN PHOSPHATE

Esterified phosphate is an integral part of some Vgs (Table 1). Engelmann, F. and Friedel, T. (1974)first identified phosphorylserine in digests *of Leucophaea maderae* Vg. While other investigators had failed to detect significant quantities of covalently attached phosphate in various other insect Vgs and Vts (Gellissen, G., *et al.*, 1976; Chen, T. *et al.*, 1978; Chino, H., *et al.*, 1977), Atlas, S., *et al.* (1978) reported that *Culex pipiens fatigens* Vt is a phosphoprotein. Using reduced and carboxymethylated Vt and specific dye staining of SDS gels, they showed that both subunits contained phosphate. $[^{32}p]NaH_2PO_4$ has been injected into vitellogenic ovariectomized *B. germanica* females, and the Vg purified from the hemolymph (Nordin, J. and Kunkel, J., unpublished studies). Samples delipidated by two different procedures had [32p]phosphate present in protein and in a glycopeptide fraction, but none could be detected in direct linkage with mannose. *Bombyx mori* Vt has its large subunit phosphorylated more rapidly than its small subunit, suggesting a differential access by an endogenous kinase (Takahashi, S., 1980).

Whether phosphate is localized in a single region of insect Vg and Vt polypeptides as it is in the phosvitin segment of vertebrate Vgs (Redshaw, M. and Follett, B., 1971; Gottlieb, T. and Wallace, R., 1981; Bergink, E. and Wallace, R., 1974; Christmann, J. *et al.*, 1977) is not known. However, since various aspects of Vt structure and function may be related to its content and/or distribution of phosphate, localizing this component is an important area for future research.

2.3 Lipophorin

Knowledge of the lipophorins (Lps) is based primarily on studies of their structure and function in the hemolymph (Chino, H., *et al.*, 198la; see Chino, H., vol. 10). However they are a major "contaminant" of insect vitellin preparations and may be important contributors to the stored nutriments in eggs of certain taxa such as *Bombyx mori* (Irie, K. and Yamashita, O., 1980). Lps have been compared to Vt and declared similar with respect to amino acid composition, polypeptide size and proteolytic sensitivity, and immunological properties (Harry, P. *et al.*, 1979; Pattnaik, N. *et al.*, 1979). These assertions have been subjects of debate and will be examined below. It is also probable that the Lps play important physiological roles in all insects prior to ovulation, in aiding the transport of lipids to the oocyte (Chino, H. *et al.*, 1977) and perhaps after ovulation within the developing egg by providing the embryo with access to stored lipid in the yolk. With these possibilities in mind the known structures and function of Lps will be discussed and related to our present understanding of egg physiology.

Lipophorin, as its name implies, is the major lipid transport protein in insects. This conclusion is based on the efforts in several laboratories over the last 20 years. Stimulation of triglyceride mobilization in the fat body first results in its conversion to diacylglycerol which then specifically complexes with Lp and moves through the hemolymph to specific sites of utilization such as flight muscle, ovary and intestinal tissue (Chino, H. *et al.*, 1981 b; Chino, H., vol. 10).

2.3.1 LIPOPHORIN PROPERTIES

Synthesis of LP occurs in the fat body (Peled, Y. and Tietz, A., 1975) and many of the details of this

process were uncovered by Gellissen, G. and Wyatt, G. (1981) using *Locusta migratoria* as the model system. These investigators conclude that Lp is synthesized as an 85K polypeptide without post-translational modification. Multiple peptides in that weight range were observed in *Blattella germanica* (Kunkel, J. and Pan, M., 1976) for a protein, SP-II, now recognized as a Lp. This suggests multiple independent genes or variable post-translational processing for the Lp of that species. Gellissen, G. and Wyatt, G. (1981) could not obtain electrophoretic evidence for any higher molecular weight (> 85K) peptide precursors of Lp. They suggest that higher molecular weight subunits of Lp reported for other species (Table 1) (Pattnaik, N., *et al.,* 1979; Chino, H., *et al.,* 1981b; Gellissen, G. and Emmerich, H., 1980) are artifacts possibly brought about during a precipitation stage of the protein purification. The fact that the aggregates are not solublized by sulfhydryl reducing agents and SDS suggests the possibility that cross-linking occurs. The greater proteolytic sensitivity of the putative aggregates and the insensitivity of the 85K monomer (Pattnaik, N., *et al.,* 1979) could be interpreted as a partial denaturation of the aggregated protein rather than an internal location for the 85K subunit.

There appear to be only minor changes in hemolymph Lp titer during vitellogenesis (Gellissen, G. and Emmerich, H., 1978) and, in contrast to that of Vg, its synthesis is marginally stimulated in both sexes by juvenile hormone (Gellissen, G. and Wyatt, G., 1981; see Koeppe, J. and Fuchs, M., vol. 8).

Observations of lipid release from insect fat body and its complexing with hemolymph protein were first reported two decades ago (Siakotos, A., 1960; Tietz, A., 1962). Chino, H. and Gilbert, L. (1965) first showed that while triglyceride is the major neutral lipid reserve in fat body, diglyceride is actually transported. They proposed that a hemolymph protein diglyceride complex provided the mechanism for lipid transport in insects. Subsequent isolation and characterization studies showed that the major contributing species in this process is a high-density lipoprotein class (HDL) in both *Hyalophora cecropia* and *Hyalophora gloveri* (Thomas, K. and Gilbert, L., 1968, 1969). Only recently has general agreement been reached that this class of molecules be termed lipophorins (Chino, H., *et al.,* 1981a). An extensive comparison of Lp from pupal hemolymph (Chino, H., *et al.,* 1969) and eggs (Chino, H., *et at.,* 1977) provides considerable insight into the role of this protein. Chemical and physical characterizations of the two molecules suggest they both contain the same apoprotein but the lipid composition of the egg form, specifically the diacylglycerol content, is only a few percent of the hemolymph protein. Both egg and hemolymph forms were found to be glycoproteins. Using highly purified proteins which subsequently were identified as Lp and Vg, Chino, H. and co-workers (1969) showed that Lp can accept [^{14}C]diacylglycerol from prelabeled fat bodies *in vitro.* Vg was virtually inactive in this respect. Another experiment showed that the *in vitro* uptake of diacylglycerol by Lp could not occur in the absence of a donor tissue (in this case fat body). This later point is critical to keep in mind if a physiological function for Lp is to be sought in the early embryo.

Both egg and hemolymph Lps accept diacyl-glycerol from fat body and have identical amino acid compositions, configuration and electrophoretic mobility. Chino, H. and co-workers (1977) concluded that the egg-bound Lp derives from the hemolymph and is probably entrapped in the egg prior to chorion formation by a non-specific uptake mechanism. Calculations by the authors ruled out any selective sequestration of hemolymph Lp by the egg, as occurs with Vg. This may also be true of the Lp of *B. germanica* which is taken up at a low but significant rate (Kunkel, J. and Pan, M., 1976) and accumulates as a storage product. However, another *B. germanica* serum protein, SP-I, is either excluded from uptake or destroyed soon thereafter, since it does not accumulate in the developing oocyte. A similar destruction has been postulated for the apparent exclusion of a major serum protein from the oocytes of *Locusta migratoria* (Lange, A. and Loughton, B., 1981). Bearing in mind that Lp may have an important function in the early embryo, more attention should be given to its sequestration, even if it is at a dramatically lower rate than that of Vg.

2.3.2 LIPOPHORIN AMINO ACID COMPOSITION

The protein moiety of Lp has an amino acid composition suggested to be close to that of Vg

(Gellissen, G., *et al.,* 1976; Mundall, E. and Law, J., 1979). Despite superficial similarities which are shared with many diverse proteins (i.e. high Asx and Glx and low Met; Hagedorn, H. and Kunkel, J., 1979) this suggestion does not stand up to close scrutiny. Careful inspection of the individual amino acid compositions, or more conveniently, by singular value decomposition, SVD (Nash, J., 1979), shows that the Lps stand apart as a group distinct from the Vts (Fig. 3). The distinctive features which make Lps different from Vts, and which are resolved by SVD 2 (Table 2) include a very low methionine content (all lower than any Vg) as well as distinctively high glycine, leucine and valine contents. Although the significance of the low met content is not clear, it is evident that the elevated Gly, Leu and Val contents would aid in creating lipid binding domains consistent with Lp's function.

When the Lp amino acid compositions are clustered using the Fitch-Margoliash tree-building algorithm (Fitch, W. and Margoliash, E., 1969), the Lps are connected by a branching pattern consistent with their taxonomic relationships and distinctly separated from the Vgs and Vts (Fig. 4). It is also evident from the limb lengths of the tree that the observed compositional differences between the Lps are smaller than the differences seen between the Vts. This is consistent with immunological evidence. The Lps of cockroaches cross-react across the order Dictyoptera (Kunkel, J., unpublished results), whereas the cockroach vitellins are limited in their cross-reaction outside intrageneric levels.

The suggested similarity of Vts and Lps bears on the crucial evolutionary and physiological question: have Vts had multiple origins during arthropod evolution (Thomson, J., 1981)? The observation of large and small peptides for a number of Lps added an additional parallel between Lp and Vt. However the suggestion (Gellissen, G. and Wyatt, G., 1981) that the large peptide of Lp, when observed, is an artifact of crosslinking is consistent with distinct evolutionary origins for Lp and Vt. If the Lps have given rise during evolution to the Vts then it probably occurred prior to the divergence of the holometabola and hemimetabola. Lps from both these later groups have similar compositions and the Vts from each of these species for which we have
amino acid analyses Fall into the same Vt class (i.e. those with a high molecular weight precuros which is processed into circa 100K and circa 50K peptides in the secreted Vg; class I of Harnish, D. and White, B., 1982). Considering the striking finding of Irie, K. and Yamashita, D. (1980) that *Bombyx mori* eggs can survive and hatch out healthy larvae by using alternative proteins to form yolk, it would be wise to continue a search for instances in which a predominant or essential yolk protein has been derived from a non-female specific serum protein. So far there is no conclusive evidence that Lp is such a protein.

2.3.3 LIPOPHORIN CARBOHYDRATE

Of the four Lps characterized with respect to carbohydrate (Table 1), Man and GlcNAc are the only sugars reported. However the total saccharide composition in each protein is quite low if one compares them to Vgs and Vts. At this time it is only possible to speculate, but the sugars are probably present as high-mannose oligosaccharides. Whether all Lps will prove to be glycoproteins must await their characterization from additional species. It should be noted that cockroach Lp does not form a precipitin band with concanavalin-A (Kunkel, J. *et al.,* 1981). Thus if a high-mannose containing oligosaccharide is present in this organism's Lp there is probably only one chain per native Lp molecule, a circumstance which would make it behave as a hapten, not capable of forming a precipitate.

2.3.4 LIPOPHORIN LIPID

Since Lp's main function is diacylglycerol transport it is not surprising to find it enriched in this lipid class. Measurements of the stored chemical form of diglyceride by both Tietz, A. and Weintraub, H. (1980) and Lok, C. and Van der Horst, D. (1980) revealed that in *Locusta migratoria* it is exclusively the sn 1,2 configuration. The diacylglycerol content of the Lps is greater than the Vgs, a fact which reflects the major function of the former. However at least a passive role for Vg in lipid transport to the embryo (discussed above) cannot be overlooked.

The two Lps which have been analyzed, like Vg, contain abundant phospholipids (Table 3). The physical and chemical properties of *M. sexta* Lp have been compared with its high-density

Lipoprotein counterpart in mammals (Pattnaik, N., *et al.*, 1979). The most obvious contrast is that the mammalian lipoproteins contain a high percentage of triglycerides and cholesteryl ester, while these are much reduced in *M. sexta* Lp (and other insect Lps also). The *Manduca sexta* Lp apoprotein displays size and solubility properties which closely resemble the apoprotein portion of mammalian low-density lipoproteins. The lipid difference is a clue that the physiological mechanism of insect lipid transport proteins is different from their mammalian counterparts, reflecting different physiological environments (Pattnaik, N. *et al.*, 1979). Proposed localization of diacylglycerol on the outer surface of M. *sexta* Lp provides for its rapid and reversible uptake from various tissues, allowing Lp to serve as a "recyclable" shuttle (Gilbert, L. and Chino, H., 1974). In contrast, mammalian carrier lipoproteins are endocytosed and degraded when yielding their lipid to accepting tissues.

Evidence for dynamic interactions between two Forms of *L. migratoria* Lp are reported to occur (Van der Horst, D., *et al.*, 1981). One form is a multimer of the protein-A, yellow. Labeling experiments suggested that both of these forms appear to rapidly exchange [^{14}C]diacylglycerol. Interactions

with additional proteins were noted upon injection an adipokinetic hormone (Beenakkers, A. *et al.*, vol. 10). The observed rapid exchange of lipid between the various protein forms suggests that the polar diglyceride is probably carried on the outer surface of the proteins. Such a location would be consistent with its function as a lipid "shuttle". However, as mentioned above, its isolation from yolk (Chino, H., *et al.*, 1977) and its uptake into oocytes (Kunkel, J. and Pan, M., 1976) indicate it may also serve as a yolk precursor.

3 SYNTHESIS OF YOLK PROTEIN

The synthesis of major yolk proteins is of interest for a number of reasons. Vitellogenesis in vertebrate liver and insect fat body are being proposed as models of hormonally regulated gene expression (Tara, J. and Smith, D., 1979; Wyatt, G., 1980). Also, development of fat body competence to res pond to hormonal cues by Vg synthesis has been suggested as a minimal model of metamorphosis (Kunkel, J., 1981). Currently investigators are establishing the diversity and extent of phenomena which are encompassed by the hormonal induction of Vg. This includes initial hormone titer and receptor studies, nucleic acid involvement, protein, oligosaccharide, lipid, phosphate and energy considerations.

3.1 Hormonal induction of vitellogenin

The initial interaction, of the vitellogenic hormones with their receptors, and how that interaction results in a response, are both at a primitive stage of understanding in insects. Although 20-hydroxyecdysone was the first hormone to be shown to interact rapidly with certain "sensor" genes in the regulation of molting, its debatable role in vitellogenesis (Fuchs, M. and Kang, S.-H., 1981; Bownes, M., 1982) of some species is not understood mechanistically. The ecdysone receptor has not been studied with reference to the vitellogenic process (see Jund, M. and Osterbur, D., vol. 7). The JH "receptor", on the other hand, has been studied in a number of insects: in the fat body *of Leucophaea maderae* (Engelmann, F., 1981), *Blaberus discoidalis* (Kunkel, J. and Wojchowski, D., 1981) and *Locusta migratoria* (Wyatt, G., 1980) and in the epidermis of *Drosophila hydei* (Klages, G. *et al.*, 1980; see Goodman, W. and Chang, E., vol. 7). Work on the fat body concurs that a high-affinity cytoplasmic juvenile hormone (JH) binding factor exists whose presence is correlated with the capacity of the tissue to synthesize Vg. Whether this factor works similarly to the vertebrate steroid hormone receptors is under current study. The subject is somewhat obscured by the presence in fat body extracts of other JH binding components of lower affinity, one of which has been ascribed to contamination of the extracts with hemolymph JH-carrier protein. This carrier protein, which is synthesized in the fat body (Nowock, J. *et al.*, 1975), plays a documented protective role, shielding hemolymph JH from hydrolysis by hemolymph esterases (see Hammock, B., vol. 7). The present decade will hopefully see a clarification of the detailed roles which the receptor and carrier, as well as JH esterases, of the hemolymph and fat body play in regulating insect vitellogenesis.

3.2 Vitellogenin protein synthesis

The synthesis of the protein component of Vg involves the general protein synthetic machinery for secreted proteins, i.e. a source of mRNA, tRNA, and amino acids to interact at the rough ER (see Kaulenas, M., vol. 10). The study of Vg mRNA is being aided by its abundance at the peak of Vg synthesis. In *L. migratoria* it is visible in methyl-Hg gels of whole RNA extracts of vitellogenic fat body (Chinzei, Y., *et al.*, 1982). It is the abundance of the message which apparently accounts for the preponderance of Vg in the secretory product of activated fat bodies. No selective amplification of the Vg genes has been reported, in contrast to the chorion genes which are amplified 12 to 60 fold in the follicle cells of *Drosophila* shortly prior to the 2 hour chorion deposition phase (Ish-Horowitz, D., 1982). This difference may be due to the fact that the Vg genes of the fat body, nurse cells and follicle cells will be active for a relatively long period of time and the dominance of Vg synthesis depends more on the stability of the Vg mRNA than on its precise timing of appearance.

The existence of multiple, non-allelic Vg genes and/or Vgs has been reported in most insect and vertebrate systems in which it has been sought. This complicates somewhat all-subsequent studies since it must be assumed that a mixture of mRNAs and peptides is to be expected when Vg is induced. This complexity must be dealt with analytically at some point. Currently there is only one species of insect, *Leucophaea maderae*, on which there is considerable work. yet no evidence for multiple Vg gene products. Even in this species, Vg is perhaps composed of multiple high molecular weight precursors since the variety of polypeptides in its processed form add up to more than 300K Mr (Koeppe, J. and Offengand, J., 1976). One problem with all of the type I Vgs with high molecular weight precursors is that small processing steps are obscured by the large size, and also the handling of the high molecular weight mRNAs becomes a very delicate operation (Wyatt, G. R., personal communication). For this reason considerable work is rightfully being done on *Drosophila melanogaster* whose Vg genes are more manageable in size. For instance, each of the *Drosophila* Vgs is first synthesized as a pre-Vg which has a leader sequence processed off, resulting in a measurable difference (ca. 1000 M_r) between the nascent peptides and the secreted product peptides (Warren, T., *et al.*, 1979). Such a small difference between a pre-Vg and a Vg would probably be missed in the larger precursors of group I and group II. Another simplicity of the Vgs of the Drosophilidae is the limited glycosylation that they undergo; only YP-2 of the three Vts appears to be glycoslylated (Mintzas, A. and Kambysellis, M., 1982).

The primary response of fat body cells to JH involves a longer delay until Vg is secreted than occurs in a secondary response of the tissue to the hormone (Koeppe, J. and Fuchs, M. vol. 8). At least two different delay phenomena may be operating, obscured because the kinetics of Vg accumulation were recorded differently in the two systems studied so far. The delay of initial appearance of Vg as a secretion product is focused on in *Blattella germanica* and represents a 16 hour delay for the primary response but only a 6 hour delay for a secondary response to JH (Kunkel, J., 1981). It is possible that this same short-term difference in response is operating in *Locusta*, however, the published data focus on the longer-term accumulation pattern of the Vg secretion cycle (Chen, T., *et al.*, 1978). This longer-term pattern of secretion may be modulated by a number of factors including possible Vg mRNA storage (Chinzei, Y., *et al.*, 1982), DNA ploidy (Nair, K., *et al.*, 1981) and rough ER availability (Della-Cioppa, G. and Engelmann, F., 1980) all of which may be different at the time of a secondary response. These data suggest that there is a range of phenomena contributing to the differences between primary and secondary responses. Future studies could find this paradigm useful in elucidating the actions of JH (see Riddiford, L., vol. 8).

3.3 Carbohydrate biosynthesis

Although the high-mannose oligosaccharide is the major if not exclusive form of carbohydrate on insect Vgs and Vts, the presence of minor amounts of O-glycosidically linked saccharides cannot be excluded since experiments to analyze for them have not yet been conducted.

Because *Xenopus laevis* Vt contains the complex type of oligosaccharide (Gottlieb, T. and Wallace, R., 1982) and both high-mannose and complex

types of oligosaccharides from all biological sources examined to date share the common precursor Glc_3-Man_9-$GlcNAc_2$ pyrophosphoryl dolichol (Struck, D. and Lennarz, W., 1980), this compound is probably also involved in Vg oligosaccharide biosynthesis. The previously noted tunicamycin inhibition of *B. germanica* Vg secretion and *X. laevis* Vg glycosylation (Gottlieb, T. and Wallace, R., 1982) plus the identification of Glc_1 Man_9-$GlcNAc_2$ as a constituent of mature Vt of B. *germanica* (Nordin, J. and Kunkel, J., 1982) give further support that this type of pathway is operative in Vg glycosylation.

No direct evidence has been obtained regarding the mechanism of oligosaccharide processing of insect Vgs, although it is presumed to follow after the transfer of the glucosylated high-mannose oligosaccharide from dolichyl pyrophosphate to protein (Struck, D. and Lennarz, W., 1980; Hubbard, S. and Ivatt, R., 1981). Following donation of the oligosaccharide to polypeptide (presumably in the rough endoplasmic reticulum) any modification of the attached saccharides would be catalyzed by specific glycosidases (Hubbard, S. and Ivatt, R., 1981). The large size and heterogeneity of oligosaccharides from mature Vts of insects (Nordin, J. and Kunkel, J., 1982: Butters, T., *et al.*, 1981) and the observed processing of *Xenopus laevis* oligosaccharide in the smooth endoplasmic reticulum (Gottlieb, T. and Wallace, R., 1982) rather than the rough endoplasmic reticulum, suggests that processing steps and locations typical of higher animal cells may not be found in insects and amphibians. It is also conceivable that these trimming reactions are "bypassed" because the amount of secretion product (Vg) simply overwhelms the capacity to modify the oligosaccharide chains uniformly. Alternatively, the positions of the oligosaccharide chains on the mature Vg may be such that they are "exposed" to differing degrees to the processing glycosidases.

3.4 Vitellogenin lipid biosynthesis

Lipid constituents of Vg are presumably intercalated into the hydrophobic core or bound to its surface prior to release into the hemolymph. This is one aspect of post-translational modification of Vg which has not been explored. JH has been found to stimulate the incorporation of acetate and choline into lipid (Della-Cioppa, G. and Engelmann, F., 1980) but it is not clear if this function is associated with supplying the lipid moiety of Vg or the lipids involved with the membranes of the protein synthetic and secretory apparatus. It is also unclear where and how conjugated ecdysteroid is bound to Vt (Lagueux, M. *et al.* 1981). Certainly, more information is needed regarding the possible functions of egg-bound Vt as a vehicle for transporting lipid (diglyceride, cholesterol, ecdysteroid) to the developing embryo. A number of exciting challenges regarding the incorporation of lipids into Vg and their possible roles in egg development are awaiting the field of insect biochemistry.

3.5 Vitellogenin phosphate biosynthesis

Phosphorylation of insect Vg *in vitro* has not been reported but a typical ATP-dependent protein kinase activity (Glass, D. and Krebs, E., 1980) utilizing dephosphorylated avian phosvitin as acceptor has been described and characterized (Goldstein, J. and Hasty, M., 1973). This enzyme is also capable of phosphorylating avian Vg (Christmann, J., *et al.*, 1977). The distribution of phosphoserine residues on insect Vgs relative to individual polypeptide chains, localization and characterization of kinase activities and their temporal relationships to the overall processing of Vg are areas of investigation which should prove fruitful in the future (Takahashi, S., 1980). The phosphate and carbohydrate-rich domains of Vgs may be extremely important in facilitating secretion, conformation and nutritional functions of the molecule.

3.6 Post-translational modification of vitellogenin

After leaving the ribosome, Vg starts a long journey which ends in its proteolysis and consumption by the developing embryo. During that journey the protein moiety is modified in a number of ways, including carbohydrate and phosphate attachment as well as possible temporal changes in these moieties. The array of possible changes to amino acids has barely been approached yet for Vg although *a priori* one would not expect too many irreversible modifications in the residues since they

are destined for use as a nutritive source. Two targets of modifications for which a body of information is available -- carbohydrate and protein will be discussed.

3.6.1 CARBOHYDRATE MODIFICATION

Information regarding the maturation of N-glycosidically linked oligosaccharides of glycoproteins in various systems is now well established (Hubbard, S. and Ivatt, R., 1981). Although the details of such work are obviously beyond the scope of this chapter, the essential steps as they occur in higher animal cells are described, since they have interesting ramifications when considered with insect Vg biosynthesis.

Following transfer of the completed oligosaccharide Glc_3-Man_9-$GlcNAc_2$ (Figure 5a) to an acceptor polypeptide the three glucoses and various numbers of mannose units are removed by specific glycosidases in the endoplasmic reticulum. If the final product is to be a complex type chain (Figure 5b) all sugars distal to the core region are removed (note identical core structure in a and b). Single-step additions of GlcNAc, Gal and NANA from their respective nucleotidyl donors complete the "antenna" regions of the complex chains (Hubbard, S. and Ivatt, R., 1981).

Hughes, R. and Butters, T. (1981) claim that cells of insects in general, and the mosquito *(Aedes aegypti)* in particular, lack the processing machinery of higher animals. As studies in our laboratory showed, these workers also found that synthesis of the N-glycosidically linked chains was tunicamycin-sensitive, indicating that the dolichyl phosphoryl route is involved. They also confirmed the absence of transferases for N-acetylglucosaminyl, galactosyl and sialyl residues which are needed for complex type oligosaccharide assembly. Further structural investigation concluded that this particular A. *aegypti* cell membrane glycoprotein contains only the completely unprocessed high-mannose chain (Glc_3-Man_9-$GlcNAc_2$). It is clear, however, that some insects possess limited processing machinery for their Vg oligosaccharides (Nordin, J. and Kunkel, J., 1982). Since in the cockroach a small portion of the total oligosaccharide fraction still contains one glucose residue, the mature glycoprotein has only very limited processing attending its attachment to the chain, and in this sense is a "degenerate" form of the mosquito oligosaccharide. Since both Vg and Vt oligosaccharides are size heterogeneous, the limited modifications observed are posttranslational events, probably occurring in the fat body.

3.6.2 PROTEIN MODIFICATION

During its synthesis on the ribosome the pre-Vg is vectorially transported into the cisterna of the endoplasmic reticulum (ER) and subsequently has its signal sequence clipped off (Brennan, M., *et al.*, 1980), becoming pro-Vg. This phenomenon has only been described for the dipteran Vg for which the pre-VG size is small enough to allow the loss of the signal peptide to cause an electrophoretically detectable size change. It is assumed, but unproven, that the Vgs of other species also undergo this cleavage although it is by no means an obligatory step in the vectoral transport process. Since it is assumed that a protein takes on its tertiary structure' as it arrives in the ER cisterna it may be that a signal peptide is cleaved off prior to termination of the Vg peptide synthesis.

From the point of vectoral transport on, group II and group III Vgs do not undergo any reported additional peptide cleavages until they are digested by the embryo. The larger group I Vgs, however, are reported to undergo extensive changes whose functions are unclear. Soon after synthesis, and still within the fat body, the pro-Vgs of *Locusta* and *Leucophaea* undergo a rapid but limited cleavage from the ~250K peptide size to several ~100K and ~50K peptides. This cleavage occurs in *Locusta* Vg even if the Vg m-RNA is translated in *Xenopus* oocytes (Chen, T., 1980), suggesting that the cleavage is at particularly vulnerable loops of the peptide chain as determined by tertiary structure of the newly synthesized Vg. Additional cleavage occurs in *Leucophaea* Vg after uptake into the oocyte (Koeppe, J. and Offengand, J., 1976; Masler, E. and Offengand, J., 1982) during the 14S to 28S conversion.

The postovulatory modification of *Blattella germanica* Vt is the only cleavage of Vt (or Vg) which has been shown to have an immunological change associated with it, and seems to be related to the start of Vt utilization by the embryo (Storella, J. and

Kunkel, J., 1979). This cleavage cannot be mimicked by using trypsin or chymotrypsin, and it is assumed that it involves a new cathepsin that is synthesized or released into the yolk environment at that time.

4 SECRETION

While it is clear that the insect fat body secretes Vg, regulation of its movement to the organ's exterior is not understood. Gottlieb, T. and Wallace, R. (1982) have shown that unglycosylated *Xenopus laevis* Vg is secreted in a normal manner from the liver. However, inhibition of secretion caused by absence of sugar from insect serum proteins has been reported (Miller, S. and Silhacek, D., 1982). We have noted that the Vg precursor in *B. ger-manica* accumulates, is not cleaved and is not secreted when the animal is treated with tunicamycin. However the basic structural differences which exist between vertebrate and insect Vgs make correlations of these observations with insect Vgs impossible to draw.

There are at least two functions which the carbohydrate portions of a glycoprotein play in the secretion process.

(1) The oligosaccharide may contain certain structural features which direct it during post-translational glycosylation either toward vesicle-mediated exocytosis at the plasma membrane or sequestration into lysosomes or other organelles. This "ticketing" mechanism has been elucidated mainly by E. F. Neufeld, P. D. Stahl, W. S. Sly and co-workers (Neufeld, E. and Ashwell, G., 1980). It functions to control the correct cellular placement of liver lysosomal hydrolases, which are glycoproteins. The phosphorylation of certain mannosyl residues during passage through the liver ER leads to their sequestration into lysosomes, whereas processing and formation of complex chains are preconditions favoring export from the cell (Miller, A. *et al.*, 1981).

(2) Carbohydrate may confer a certain degree of stability to the protein subunits (especially ones enriched in hydrophobic residues), ensuring proper assembly or preventing aggregation prior to secretion. Two illustrations of the need for such glycosylation come from studies with vesicular stomatitis virus (vsv) virion assembly in Chinese hamster ovary cells (Gibson, R., *et al.*, 1979) and acetylcholine receptor subunits of muscle cells (Merlie, J. *et al.*, 1982). In both cases, tunicamycin-treated cells synthesized aglycosyl protein. The metabolic fate of each was monitored and it was found that proper assembly of the receptor molecule was blocked and that virion assembly from its components did not occur when the carbohydrate portions of the glycoproteins were unattached. However, since general secretion from insect fat body is inhibited by tunicamycin (Miller, S. and Silhacek, D., 1982), the possibility must be entertained that a glycoprotein moiety in the membrane machinery for secretion is being affected.

5 UPTAKE OF VITELLOGENIN

Much contemporary interest in glycoprotein research centers on the discovery that saccharides of certain glycoproteins can play a role in their uptake by cells (Neufeld, E. and Ashwell, G., 1980). Potential for involvement of the polymannose side chains of Vg in its recognition for uptake by the oocyte arises from published reports implicating mannosyl oligosaccharides of glycoproteins in their clearance from circulation (Baynes, J. and Wold, F., 1976; Winkelhake, J. and Nicolson, G., 1976). More definitive and direct evidence for a mannosyl recognition system on pulmonary reticuloendothelial cells was provided by Stahl, P. *et al.* (1978) using rat alveolar macrophages. In these studies neoglycoproteins (i.e. non-glycoproteins with specific glycosyl groups chemically linked to them) were utilized to demonstrate the specificity of this recognition system. Roth, T. and Porter, K. (1964) first demonstrated that Vg of *Aedes aegypti* is sequestered by developing oocytes from the maternal bloodstream by adsorptive endocytosis. Such uptake by definition is a site-saturable phenomenon involving specific receptors (Roth, T., *et al.*, 1976; Yosko, S., *et al.*, 1981). Coated-pit-vesicle precursors on the oocyte plasma membrane confine clusters of receptors which bind Vg specifically. Further invagination of these regions, and their pinching off

from the plasma membrane, results in transport of Vg to the yolk granules by endocytotic vesicles (Roth, T. *et al.*, 1976). The specificity of uptake has been demonstrated in several groups of insects using labeled Vgs and control proteins (Kunkel, J. and Pan, M., 1976; Ferenz, H., 1978; Lange, A. and Loughton, B., 1981).

There is evidence that certain changes in molecular properties are coincident with Vg's uptake. Vt isolated from the eggs *of Philosamia cynthia* has a lipid composition different from that of the hemolymph protein (Chino, H., *et al.*, 1977). The changes are found primarily in certain phospholipids since the total lipid content remains constant. Chinzei, Y. *et al.* (1981) also found changes in lipid composition of Vg upon uptake by *Locusta* oocytes. It is possible that specific aggregation plays some role in concentrating the protein for deposition in the yolk granules; after uptake, the Vt of *Leucophaea maderae* is dimerized (Dejmal, R. and Brookes, V., 1972) and after chorion formation, *Blattella germanica* Vt forms a 31s Vt from its original 18s form (Storella, J. and Kunkel, J., 1979). It is also understood that freshly endocytosed insect Vt moves to the yolk granules and not the lysosomes. Perhaps the absence of phosphate on the oligo-saccharide ensures proper localization; however, the entire sequestration and compartmentation mechanism remains a mystery. Another possible function of the oligosaccharide of egg-bound Vt might be to program these molecules for degradation during embryonic development. Selective destruction has been postulated as a reason for the lack of one serum protein's accumulation in *Locusta* oocytes (Lange, A. and Loughton, B., 1981). Also, oligosaccharide seems to be stripped from the Vt of *Locusta* a few days after ovulation at a time when utilization would be expected to begin (McGregor, D. and Loughton, B., 1977). W. H. Telfer and co-workers (1981) have measured currents operant in ovarioles which are being supplied with nutrients from nurse cells. Electrical potentials are suggested to be involved in the movement of macromolecules from the nurse cells to the oocyte.

Further insight into the molecular mechanism of the adsorptive endocytosis process has recently been obtained with mammalian cells. Transglutaminase (glutaminylpeptide glutamyl transferase EC 2.32.13) plays a pivotal role in membrane reorganization, which accompanies internalization of adsorbed ligands. Rat kidney cells will internalize rhodamine-labeled α2-macroglobulin but this process is reversibly blocked by inhibitors of transglutaminase (Levitski, A. *et al.*, 1980). These studies indicate that binding of ligand to receptor causes a structural change in the receptor which leads to the irreversible trapping of the complex in the coated pit. An investigation describing Vg uptake (Tucciarone, L. and Lanclos, K., 1981) by *Xenopus laevis* oocytes also involves transglutaminase since inhibitors of the enzyme (e.g. dansyl cadaverine and methylamine) blocked Vg uptake. These compounds also blocked the movement of Vg to the yolk platelets. Carbohydrate has been postulated to play a role in protection of Vg from destruction by oocyte lysosomes following internalization and prior to uptake by the yolk platelets (Giorgi, F. and Jacob, J., 1977).

Experiments implicating the saccharide of Vg in recognition and uptake will require specific and non-destructive modification of the Vg molecule. Techniques such as endoglycosidase H digestion to remove oligosaccharide residues, or periodate oxidation to destroy their structural integrity, may be useful in approaching this problem.

6 INTEGRATION OF VITELLOGENIN GENE EXPRESSION

The study of Vg induction and processing lends itself to many basic questions in cell biology. A useful distinction has been made between induction of Vg and the effect the induction process has on the synthesis of Lp. It now behooves us to attempt to describe the constellation of genes and cellular machinery that are involved in the vitellogenic process: in the fat body the JH receptor, JH esterases, protein synthetic and oligosaccharide synthetic machinery, packaging and secretory machinery; in the oocyte the machinery of selective endocytosis and sequestration, as well as the later differential utilization of the Vt by the embryo. Focusing on Vg as a central theme will hopefully allow the ancillary genes and processing machinery to be better understood.

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ADDENDUM

Despite the apparent rapid evolutionary divergence of vitellogenins among insects as measured by immunological cross-reaction, recent evidence has recorded a remarkable homology of the Vg messenger RNAs of *Xenopus,* chicken and *Locusta migratoria* as measured by cross-hybridization under relaxed stringency (James, T. *et al.,* 1982). This homology of nucleic acids if confirmed with more rigorous sequence studies could reflect internally conserved structure not accessed by antibodies reacting with the native surface of the Vgs. Conservation of internal structure combined with more rapid evolution of surface features is consistent with other series of homologous proteins but this would be one of the most extreme cases. At the moment the selective forces that could be restricting the divergence of internal structure over such a broad span of time are unfathomable.

A theory of structural homology among the insect vitellins has been elaborated more fully and documented with new' analyses of Vt amino acid-, compositions as well as peptide sizes (Harnish, D. and White, B., 1982). However care should be taken in using composition data from their tables since numerous typographical errors exist in the new as well as old mole per cent data. This example of errors in publication of large data tables is a real problem in this era of data analysis figures such as Fig. 3 above depend on reliable sources of data for input. Errors in that input should be able to be avoided if the conventional mole per cent data are always published (instead of the raw µmoles of amino acid per mg. protein) and tables are checked by a reader for summation to 100%. With the advent of word-processing and "camera ready" tables there should be no need for a table of numbers to accrue errors due to successive transcriptions.

Immunohistochemical localization of vitellogenin in mosquito fat body was accomplished at both the light and electronmicroscopical level (Raikhel, A. and Lea, A., 1983) underlining the continued usefulness of this technique. At the light-microscope level and using electronic image enhancement, immunofluorescence was detectable at 1 hour after a blood meal.

Juvenile hormone has an effect on the processing of vitellogenin in the hemolymph of *Oncopeltus fasciatus* (Kelley, T. and Hunt, L.-M., 1982). Without JH an incompletely processed proVg appears in the hemolymph and JH subsequently induces the cleavage to a mature form which is promptly taken up into the developing oocytes.

Two laboratories have reported on physical properties of the juvenile hormone receptor of vitellogenic fat body, one in *Locusta* (Roberts, P. and Wyatt, G., 1983) and the other in *Blaberus* (Wojchowski, D. and Kunkel, J., 1983). These two laboratories have found different sedimentation coefficients for ostensibly the same molecule, 13S and 5.4S respectively for the locust and cockroach. The smaller molecule is consistent in size with vertebrate steroid hormone receptors; the larger

molecule is unprecedented in size among cytosol receptors.

Additional evidence has been presented arguing that *Manduca sexta* lipophorin does have two immunologically distinct apo-peptides, 240,000 Mr and 78,000 Mr respectively (Shapiro, J. and Law, J., 1983a). The only way the two sets of data for *Manduca* and *Locusta* (Table 1) could be made consistent would be for only one of two distinct *Manduca* lipophorin peptides to be subject to aggregation. A general peptide structure for lipophorin must be held in abeyance until this controversy is resolved. The adult form of lipophorin of both *Locusta* and *Manduca* are reported to contain an additional small peptide, 17,000 Mr, and this small subunit seems to associate with lipophorin as a result of adipokinetic hormone action (Wheeler, C. and Goldsworthy, G., 1983; Shapiro, J. and Law. J., 1983b).

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