

Why Are Green Caterpillars Green?

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Insects use camouflage coloration as a means of avoiding predation. The green color of the tobacco hornworm larvae, (*Manduca sexta*) can be separated into constituent blue and yellow components. The water soluble blue component is the biliprotein, insecticyanin. The yellow color is derived from lipoprotein bound carotenes. This lipoprotein, lipophorin, is the major lipid transport vehicle in insect hemolymph. In addition to transporting dietary lipid, lipophorin is also involved in the transport of lipophilic insecticides. Nearly all the recovered radioactivity in hemolymph from topically applied [¹⁴C]-DDT is associated with lipophorin. Lipophorin of adult *M. sexta* is larger, less dense and is associated with small amounts of a third, adult specific, apoprotein. Alterations in adult lipophorin density, lipid content and apoprotein stoichiometry can be caused by injection of the decapeptide, adipokinetic hormone.

Green is a popular color for insects. Green caterpillars abound. Who has grown tomatoes without encountering the ubiquitous tomato (and sometimes tobacco) hornworm? Its color blends so successfully with that of the host plant that finding the cause of the damage can be frustrating in the extreme. Even some adult insects are green and the green insect egg evades detection when placed on a host plant. Clearly, green coloration imparts a protective advantage to the phytophagous insect.

Insecticyanin

The remarkable matching of insect pigmentation to that of the host plant led to the idea that the pigment of host and pest were related, possibly identical. Early workers compared the green

pigments of plants and larval insects and concluded that the insects retained chlorophyll from the diet and sequestered it in their tissues for camouflage coloration (1).

The chlorophyll hypothesis was thoroughly destroyed by Lederer and Prziham in 1933, when they reported that the green color of some orthopterans could be resolved into a water soluble blue pigment and a fat soluble yellow carotene fraction (2). Subsequent work on a number of species showed that the blue color results from protein-bound bile pigments, usually biliverdin IX γ or IX α (3) (Figure 1). The yellow color results from protein-bound carotenes, which in the lepidoptera are usually lutein and β -carotene (4) (Figure 1). It is interesting to note that the green coloration of the eggs of a tropical tree frog, *Agalychnis dacnicolor*, has recently been shown to result from a mixture of biliverdin IX α and lutein (5).

Recent investigations have been focused on the identification of the protein-pigment complexes of insects. For example, in the tobacco hornworm, *Manduca sexta*, a blue biliprotein, insecticyanin, has been found in the hemolymph, epidermal cells and eggs (6). This protein was purified to homogeneity and crystallized by Cherbas (6). It was shown to be an oligomeric protein composed of 22,000 dalton subunits. The chromophore was tentatively identified as biliverdin IX γ , associated by non-covalent bonds to the apoproteins.

The structure of the insecticyanin apoprotein has recently been determined (7) (Figure 2). In comparing this protein with others that bind bile pigments, short regions of homology have emerged. Those segments may thus represent the sites of bile pigment binding to insecticyanin. The holoprotein appears to be a tetramer, as indicated by cross-linking experiments (7).

We do not know how insects produce bile pigments. Some evidence points to *de novo* synthesis (8), but it is possible that some dietary component is involved. In mammals, protoporphyrin IX is cleaved to biliverdin IX α (Figure 1), but we do not know if an analogous process leads to insect biliverdins. Mammals process large amounts of protoporphyrin IX resulting from heme degradation. As insects do not make hemoglobin for oxygen transport, their supply of protoporphyrin IX is much more limited. If one could understand the route of bile pigment synthesis in insects and disrupt it, interference with camouflage coloration might be an attainable goal.

Hemolymph Lipoprotein

The yellow carotene binding protein of *M. sexta* hemolymph is a more complicated case. Carotenes are extremely water-insoluble materials. They share this property with several other natural products including sterols, fats and hydrocarbons, all of which are important to insects. This property is also shared by many xenobiotics, including pesticides. Transport of hydrophobic materials within the aqueous compartments of living organisms, e.g. blood or hemolymph, is accomplished by lipoproteins. Extensive

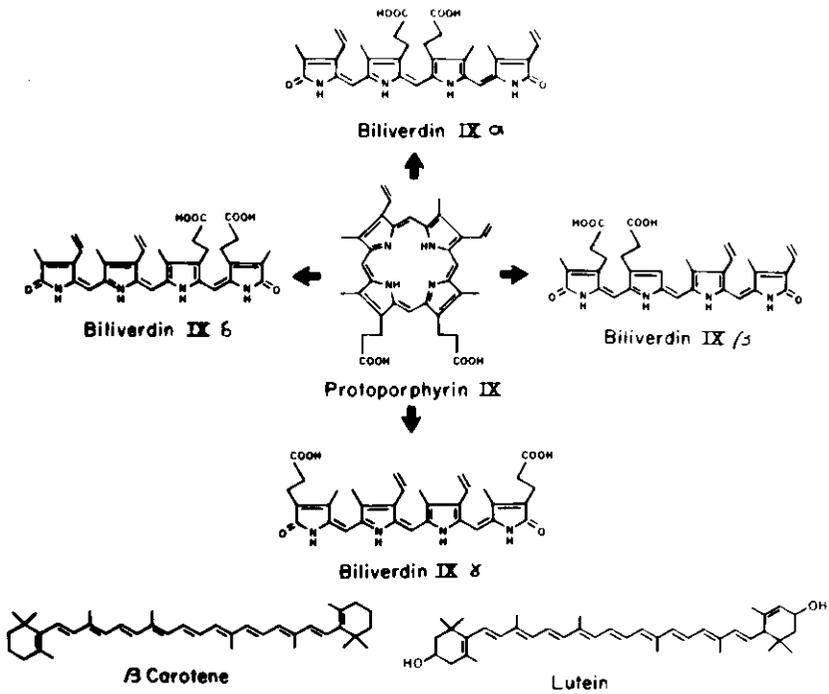


Figure 1. Bile pigments and carotenoids used by insects for coloration. The conversion of protoporphyrin IX to the various bile pigments is indicated.

10 20
 GLY-ASP-ILE-PHE-TYR-PRO-GLY-TYR-CYS-PRO-ASP-VAL-LYS-PRO-VAL-ASN-ASP-PHE-ASP-LEU-
 30 40
 SER-ALA-PHE-ALA-GLY-ALA-TRP-HIS-GLU-ILE-ALA-LYS-LEU-PRO-LEU-GLU-ASN-GLU-ASN-GLN-
 50 60
 GLY-ILE-CYS-CYS-THR-ILE-ALA-GLU-TYR-LYS-TYR-ASP-GLY-ILE-LYS-LYS-ALA-SER-VAL-TYR-ASN-SER-
 70 80
 PHE-VAL-SER-ASN-GLY-VAL-LYS-GLU-TYR-MET-GLU-GLY-ASP-LEU-GLU-ILE-ALA-PRO-ASP-ALA-
 90 100
 LYS-TYR-THR-LYS-GLN-GLY-LYS-TYR-VAL-MET-THR-PHE-ILE-PHE-GLY-GLN-ARG-VAL-VAL-ASN-
 110 120
 LEU-VAL-PRO-TRP-VAL-LEU-ALA-THR-ASP-TYR-LYS-ASN-TYR-ALA-ILE-ASN-TYR-ASN-CYS-ASP-
 130 140
 TYR-HIS-PRO-ASP-LYS-LYS-ALA-HIS-SER-ILE-HIS-ALA-TRP-ILE-LEU-SER-LYS-SER-LYS-VAL-
 150 160
 LEU-GLU-GLY-ASN-THR-LYS-GLU-VAL-VAL-ASP-ASN-VAL-LEU-LYS-THR-PHE-SER-HIS-LEU-ILE-
 170 180
 ASP-ALA-SER-LYS-PHE-ILE-SER-ASN-ASP-PHE-SER-GLU-ALA-ALA-CYS-GLN-TYR-SER-THR-THR-
 189
 TYR-SER-LEU-THR-GLY-PRO-ASP-ARG-HIS

Figure 2. The covalent amino acid structure of the blue biliprotein of *M. sexta*. Residues 60-90, 102-108 and 147-154 show some homology with other bile pigment binding proteins and may represent regions involved in chromophore binding.

studies on human serum lipoproteins have provided a model in which extremely hydrophobic materials (e.g. triacylglycerol, sterol esters, hydrocarbons) form a spherical core (9). The acyl chains of a phospholipid monolayer as well as the apolar ring system and side chain of free sterols associate with the hydrophobic core, while the polar phospholipid head groups and sterol hydroxyl groups face the aqueous exterior. Interspersed near the aqueous interface are the apoproteins, which have well defined, segregated, hydrophobic and hydrophilic regions on their surfaces. The hydrophilic regions of the apoproteins interface with the aqueous environment while the hydrophobic regions associate with the phospholipids. Based on this structural organization, the apoproteins are said to be amphiphilic. This arrangement provides for the packaging of hydrophobic material within a water compatible envelope that may be efficiently transported through the blood.

Human lipoproteins exist in several sizes and densities with differing lipid to protein ratios. These various lipoproteins have different origins in the body, different destinations and different functions (10). Thus, chylomicrons are extremely large low density particles formed in the intestine and designed to deliver dietary fat to adipose tissue. Very low density lipoproteins (VLDL), on the other hand, are smaller, more dense particles designed to deliver lipids from the liver to adipose and other tissues. Low density lipoproteins (LDL), formed from VLDL or produced in the liver or intestine deliver cholesterol to peripheral tissue, while high density lipoproteins (HDL) function to return cholesterol from peripheral tissues to the liver for catabolism. There is a complex exchange of lipids and apoproteins between the lipoprotein classes.

If one draws hemolymph from the green larva of *Manduca sexta* and mixes it with potassium bromide to a concentration of 44 percent, places this solution in an ultracentrifuge tube, overlays with saline and subjects the mixture to centrifugation at 200,000 x g for 4 hours in a vertical rotor, one resolves the green color into a lower blue phase, a clear zone and a bright yellow band in the middle of the tube (Figure 3). This is the result of a density gradient of KBr set up in the centrifugal field. Most proteins, including the blue insecticyanin, have a density greater than 1.30 g/ml and thus are sedimented to the bottom of the tube. The yellow carotene is associated with the lipoprotein of larval hemolymph, which has a density of 1.15 g/ml, and thus floats above the remainder of the hemolymph proteins.

It is thought that dietary carotene is transferred to the hemolymph lipoprotein, which is called lipophorin (11), at the midgut during digestion of food. It is transported to epidermal cells, where it probably associates with a different protein inside the cells. Unlike the blue component of green coloration, insects appear to be completely dependent upon dietary carotenes for the yellow component (4). *M. sexta* larvae, raised on a standard laboratory diet, are distinctly blue in color, rather than green.

What do we know about the structure and multiple functions of insect lipophorin? Larval lipophorin from *M. sexta* (12,13), with a density of 1.15 g/ml, is comparable to the high density lipoprotein

of human serum. Table I compares the composition of mammalian HDL with *M. sexta* larval lipophorin. The differences are in the content of diacylglycerol, a major component of lipophorin, and sterol esters, which are present in only small amounts in lipophorin. The polypeptide components are also different. Mammalian HDL contains several copies of relatively small apoproteins, the apoA series, while lipophorin contains an extremely large apoprotein, apoLp-I, of about 240,000 daltons, and a moderate sized apoLp-II of about 80,000 daltons (13). Each lipophorin particle has only one copy of each apoprotein.

Table I. Composition of High Density Lipoproteins in Insects and Man

Lipid Component	Percent Total Weight	
	Insect*	Mammals**
Triacylglycerol	0.9	2
Diacylglycerol	14.9	0.4
Sterol (% as esters)	2.0 (0%)	18 (84%)
Phospholipid	14.9	18
Hydrocarbon	4.7	--
Protein	62.7	58
Density (g/ml)	1.15	1.06-1.21

*Taken from Prasad (26)

**Taken from Chapman (27)

Xenobiotic transport by lipophorin. We believe that the function of the larval lipophorin is to transport water-insoluble materials consumed by the larva from the site of digestion and absorption in the midgut to the tissues of storage or utilization. Among these are fats, sterols and carotenes. We also know that hydrocarbons, produced in blood cells, are transported by lipophorin to epidermal cells, where they are exported to the surface of the exoskeleton (14). Lipophorins may also transport hydrophobic xenobiotics. Figure 4 shows the result of an experiment in which a sublethal dose of ^{14}C -DDT was applied to the cuticle of a fifth instar larva of *M. sexta*. After 19 hours, hemolymph was drawn and subjected to KBr density gradient centrifugation. The radioactivity in each fraction was determined, and the result can be seen. The amount of radioactive DDT in the hemolymph represented only about 0.5-1 percent of the applied dose, but virtually all of the radioactive material was associated with the lipophorin. While the mode of transport of insecticides in insects is a matter of some controversy (15) it can be clearly stated that if DDT gets into the hemolymph, it associates with lipophorin. Earlier workers (16-18) have mixed labeled insecticides with hemolymph and shown that they become associated with lipoproteins, but none of these reports identify the lipoproteins in the hemolymph.

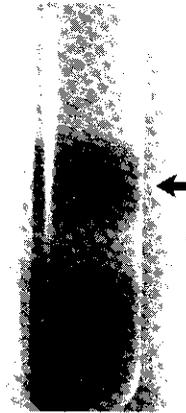


Figure 3. Potassium bromide density gradient ultracentrifugation of *M. sexta* hemolymph. The less dense yellow colored lipophorin floats above the layer of more dense ordinary proteins, including the blue insecticyanin. Arrow designates position of lipophorin in the gradient.

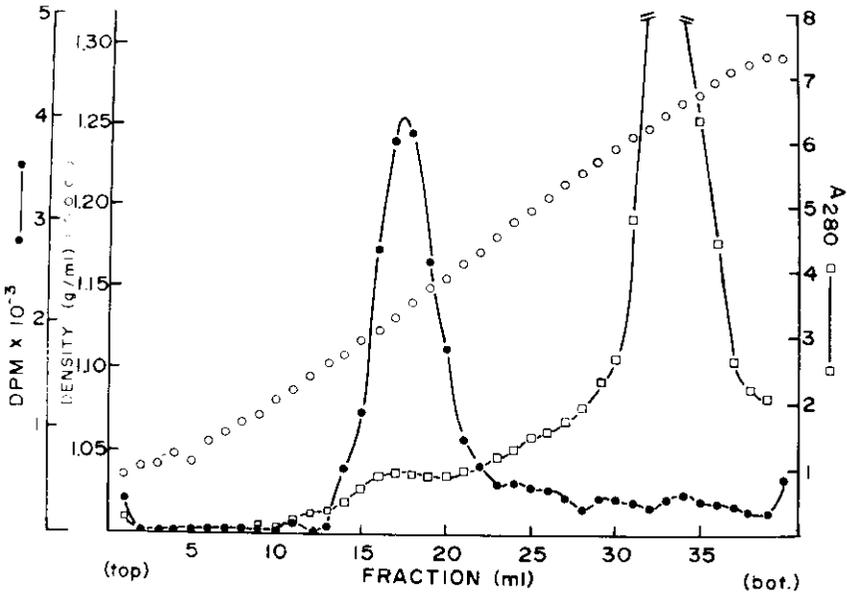


Figure 4. Distribution of [¹⁴C]-DDT in larval *M. sexta* hemolymph. 19 h after topical application, hemolymph was subjected to density gradient ultracentrifugation as shown in Figure 3. Following centrifugation the tube was fractionated and the radioactivity in each fraction determined. Most of the labeled pesticide was found in the lipophorin fraction.

Adult Lipophorin

When the larva undergoes metamorphosis to an adult moth, a somewhat different lipophorin is found in the blood. The density of this adult form is 1.08 g/ml, and a new apoprotein, apoLp-III, 17,000 daltons (19), associates with the lipophorin, in addition to apoLp-I and apoLp-II. Analysis shows that the particle has twice the lipid content as the larval form and an apoprotein ratio of 1 apoLp-I to 1 apoLp-II to 2 apoLp-III.

ApoLp-III is one of the most abundant proteins of adult hemolymph, reaching a concentration of 17 mg/ml. In hemolymph apoLp-III can be found free or associated with lipophorin. Only a small part of the total apoLp-III is associated with lipophorin when the animal is resting.

A major difference between metabolism of the larva and that of the adult is that connected with flight in the latter. Some adult insects (e.g. flies and honeybees) use sugar to fuel flight, while others, particularly those that fly long distances, use fat for flight (e.g. butterflies, moths and locusts). A few insects (e.g. tsetse flies and Colorado potato beetles) have an unusual reliance on proline as a flight fuel (20). In the case of fat utilization, it is necessary to transport large amounts of fat from the reservoirs in the fat body to the flight muscle. It is well established in the locust, Locusta migratoria, that fat is transported as diacylglycerol associated with lipophorin (20).

The process of fat mobilization for use in flight metabolism in L. migratoria is initiated by the release of a decapeptide, the adipokinetic hormone (AKH) from the corpus cardiacum (20), a gland posterior to the brain and a part of the neuroendocrine system. Similar polypeptide hormones are probably found in all adult insects and are involved in preparing the animal for flight. In the cockroach, AKH causes mobilization of carbohydrate in the form of the disaccharide, trehalose, which is produced in the fat body from the glycogen reserves and transported through the hemolymph (20). In the Colorado potato beetle Leptinotarsa decemlineata, AKH stimulates production of proline by the fat body (21). Since synthetic locust AKH causes these effects in all of these animals, it is likely that each has a similar and homologous polypeptide hormone that signals the onset of flight metabolism.

In the locust, AKH is thought to act upon receptors in the fat body cell membrane to activate adenylyl cyclase, which then activates the enzymatic machinery to convert triacylglycerol to diacylglycerol. The exact nature of that enzymatic machinery is unclear, but there is an obvious parallel to the action of glucagon on mammalian adipose tissue. When diacylglycerol leaves the locust fat body, it is accepted by locust lipophorin in the hemolymph, and at the same time, a small polypeptide, called "C protein" associates with the diacylglycerol loaded lipophorin (22). We believe that M. sexta apoLp-III is analogous to locust C protein.

To test this hypothesis, we carried out experiments in which we injected synthetic locust AKH into adult M. sexta. We observed a dramatic shift in the density of the adult lipophorin from

1.08 g/ml to 1.03 g/ml, or into the LDL class of lipoprotein (23). This change was accompanied by a large increase in size, diacylglycerol content and apoLp-III content (Figure 5). It can be seen that when most of the lipophorin has been loaded to capacity, the free apoLp-III in the hemolymph is greatly depleted.

ApoLp-III has been isolated and characterized (19). It is devoid of cysteine and tryptophan, and contains only one tyrosine residue. It is poor in glycine and proline, which tend to destroy helical conformation, and rich in leucine, glutamate and lysine, which are good helix formers. In keeping with this composition, the circular dichroism spectrum indicates a high content of helix. In addition, viscosity experiments, as well as studies on monolayers of apoLp-III at the air-buffer interface suggest that apoLp-III is a compact molecule, a characteristic of proteins with a high content of α -helix. The N-terminal sequence can be arranged into a perfect amphiphilic helix and would provide an excellent lipid binding site. Indeed, current experiments show apoLp-III to be an excellent lipid binding protein that binds either to phospholipid or diacylglycerol coated surfaces with high affinity.

Larvae lack the ability to load lipophorin with diacylglycerol, even when apoLp-III and AKH are supplied. On the other hand, larval lipophorin is readily converted to the adult form and loaded in the adult when AKH is supplied. If a foreign larval lipophorin, that from the honeybee *Apis mellifera*, is injected into adult *M. sexta* along with AKH, the foreign lipophorin is partially loaded. However, immunoprecipitation experiments with anti-apoLp-III antibodies indicate that apoLp-III did not associate with the honeybee lipophorin (24). This suggests that apoLp-III may recognize some feature of the apoproteins for binding and is not simply associating with exposed lipid.

Lipophorins from several orders of adult insects have been examined, and so far only in *L. migratoria* (22), *M. sexta* and a hemipteran, *Leptoglossus zonatus* (25), has apoLp-III been observed. Efforts are now underway to determine if apoLp-III is invariably associated with flight metabolism fueled by fat.

As one can see the question posed by the title can be answered at several levels. Pursuit of the question leads into the basic biochemistry and physiology of the insect and reveals fundamental facets of the transport of vital hydrophobic materials throughout the insect system. An understanding of the structure and functions of the lipoprotein transport vehicle may lead to a better understanding of normal physiology, as well as the mechanism for distribution of hydrophobic xenobiotics.

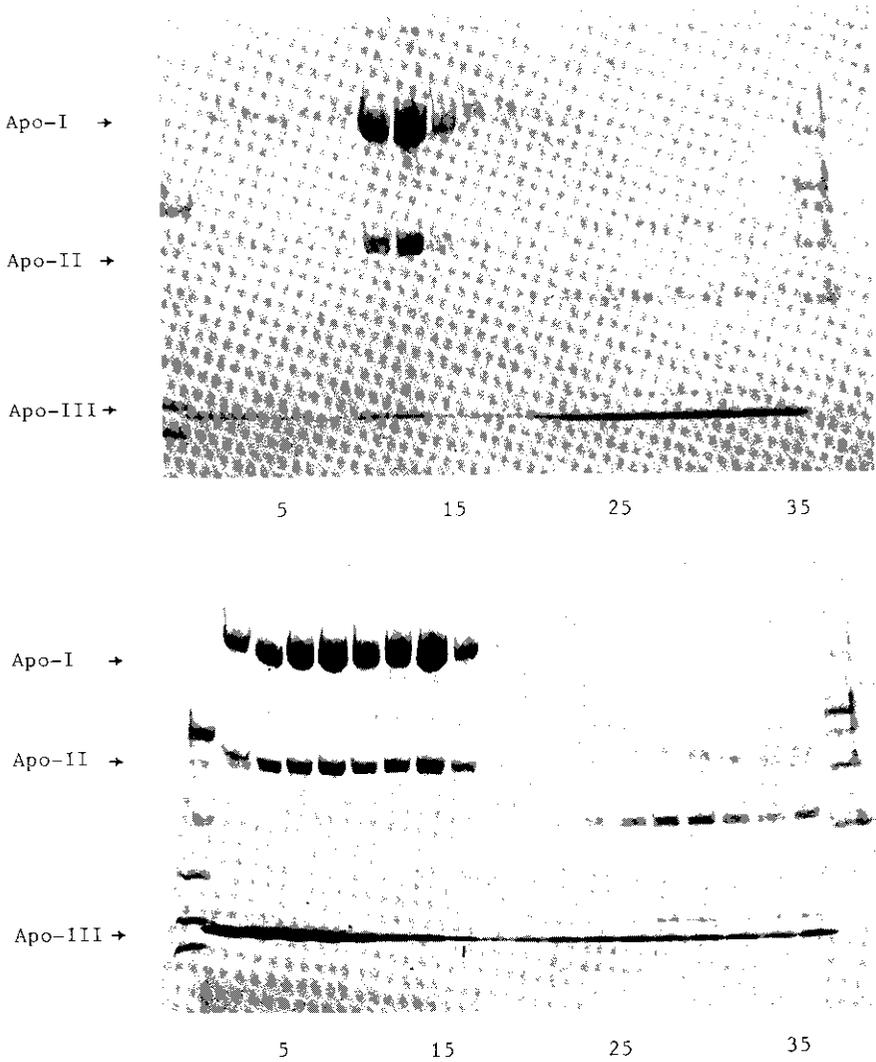


Figure 5. Effect of adipokinetic hormone on lipoporphin. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (4-15 percent acrylamide gradient slab) of *M. sexta* adult hemolymph following density gradient ultracentrifugation. Centrifuge tubes were fractionated and aliquots applied to the gel. Above, saline injected control animals; below, adipokinetic hormone (200 pmoles/animal) injected. Reproduced with permission from Ref. 23. Copyright 1983 Academic Press.

Acknowledgments

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