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26 September 1963

Diglyceride Release from Insect Fat Body: A Possible Means of Lipid Transport

Abstract. *Lipid is released in the form of diglyceride from the fat body of the cecropia silkmoth* (Hyalophora cecropia), *in both adults and pupae. This diglyceride, in the form of a complex with hemolymph protein, is the most probable means by which lipid is transported in this insect.*

Although lipids are of vital importance to many insects as metabolic fuel, little is known about the transport of lipids from the organ of storage (fat body) to sites of utilization. This report indicates that in pupae of the American silkworm (*Hyalophora cecropia*), long-chain fatty acids are transported in the form of diglycerides which are probably conjugated to hemolymph (blood) proteins.

When palmitic acid-1-C^{14} was injected into cecropia pupae and the lipid extracted from the pupa a few hours later, almost all the recovered radioactivity was found in the neutral fat fraction. The pupae used in these experiments were held at 6°C for about 6 months. Morphological examination revealed that they had not begun adult development at the time of the experiments, which took place on the day the animals were returned to room temperature. Judging by previous experience, these pupae were ready to begin development. Palmitic acid was injected as the potassium salt, albumin complex (*1*), and radioassays were performed with a liquid scintillation counter (*2*); internal standards were used to correct for quenching. When lipid was extracted separately from the fat body and hemolymph (blood), it was noted that the specific activity of the neutral fat in the hemolymph was more than 120 times greater than that in the fat body. This suggested that either the hemolymph is capable of synthesizing neutral fat from unesterified fatty acid or that the lipid is synthesized in the fat body and released into the hemolymph. Our subsequent finding, that hemolymph alone has only a negligible capacity to incorporate labeled palmitate into neutral fat, lends credence to the latter hypothesis.

To investigate the premise that the fat body releases newly synthesized lipid into the hemolymph, the following experiments were conducted. The total amount of labeled lipid extracted from the fat body and hemolymph was subjected to column chromatography (*3*). Results in Table 1 reveal that the radioactivity of the diglyceride fraction in the hemolymph was extremely high, both in total and specific activity, but that the triglyceride fraction had a very low specific activity. That the palmitate was converted to neutral fat in the fat body was demonstrated in the following manner. When isolated pupal fat body was incubated in saline (0.86 percent KCl in phosphate buffer, *p*H 6.7) containing labeled palmitate, there was a rapid incorporation of the fatty acid into neutral fat within 60 minutes. About 80 percent of the labeled neutral fat was in the form of diglyceride, while the remainder was triglyceride. However, of the total neutral fat found in this organ, almost 98 percent was triglyceride and less than 2 percent diglyceride. The specific activity of the diglyceride was almost 200 times greater than that of the triglyceride. These experiments in vitro suggest that palmitate is first incorporated into diglyceride by the fat body, and that a major fraction of the diglyceride is then released into the hemolymph. The remaining diglyceride in the fat body could conceivably be used for triglyceride synthesis by conventional biosynthetic means (*4*). Little, if any, of this triglyceride is released into the hemolymph. According to this view, one would expect the observed higher specific activity of neutral fat to be in the hemolymph rather than in the fat body.

To examine this hypothesis further, 1.5 g of fat body carefully dissected from a pupa was rinsed several times with saline solution and then incubated in 3 ml of saline solution containing 1 million count/min of palmitate (0.04 μmole), at 25°C. After 60 minutes the fat body was rinsed several times to remove excess fatty acid, and this "prelabeled" fat body was then incubated in cell-free hemolymph. The incubation media used in control experiments included saline or various albumin solutions. At the end of the incubation period, the lipid was extracted from the incubation medium with a mixture of isopropanol, heptane, and 1*N* H_2SO_4 (40 : 10 : 1 vol/vol) (*5*), and chromatographed on florisil as before. As shown in Table 2 and Fig. 1, the labeled diglyceride was rapidly released from the fat body into the hemolymph while little triglyceride was released. An appreciable quantity of free fatty acid was also released from the fat body into the hemolymph. While the release of labeled diglyceride appeared to be specific for the insect's own hemolymph, the triglyceride and unesterified fatty acids entered the albumin-containing media with the same facility as they entered the hemolymph. From these experiments, there is little doubt that the diglyceride fraction of the neutral lipid is important as a means of transporting lipid from the fat body to other metabolic sites.

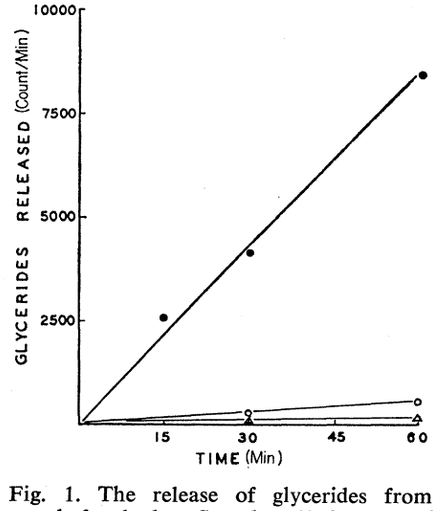

Fig. 1. The release of glycerides from pupal fat body. Samples (300 mg) of labeled fat body containing 19,900 count/min of triglyceride and 97,300 count/min of diglyceride were incubated with 1 ml of saline and 0.2 ml of cell-free hemolymph containing 1 μmole of glutathione (to inhibit blood tyrosinase) or with 0.8 ml of saline and 1 μmole of glutathione without hemolymph. Solid circles, diglyceride released into hemolymph; open circles, diglyceride released into saline; and triangles, triglyceride released into hemolymph.

Table 1. Radioactivity of labeled lipid extracted from pupal hemolymph and fat body. Potassium palmitate-1-C^{14} (0.04 μmole), with 1 million counts per minute, was injected into a male cecropia pupa and the hemolymph and fat body were collected separately 3 hours later. The following solvent sequence was used in the florisil column (*3*): hexane (hydrocarbons); 5 percent ether in hexane (cholesterol esters); 15 percent ether in hexane (triglycerides); 25 percent ether in hexane (sterols); 50 percent ether in hexane (diglycerides); 2 percent methanol in ether (monoglycerides); 4 percent acetic acid in ether (unesterified fatty acids). No radioactivity was detected in any fractions other than those listed in this table.

Lipid fraction	Hemolymph (1000 mg)			Fat body (400 mg)		
	Amount (mg)	Total counts (per minute)	Specific activity (count/min per mg)	Amount (mg)	Total counts (per minute)	Specific activity (count/min per mg)
1. Triglyceride	1.3	253	194	56	10,468	177
2. Diglyceride	2.4	121,060	50,440	1.1	5,440	4,950
3. Monoglyceride	<0.5	26		<0.5	230	
4. Unesterified fatty acid		1,508			2,754	
5. Neutral fat (sum of 1, 2, and 3)	4.2	121,339	30,330	57.6	16,138	280

A crucial question, however, concerns the validity of the chromatographic procedure used in separating a "pure" diglyceride fraction. In the procedure that we used, the triglyceride and diglyceride fractions are separated in the elution sequence by the sterol fraction. By using known compounds, we tested the column repeatedly and found the results valid. In a few instances there was some overlap between the sterol and diglyceride fraction, but never between the triglyceride and diglyceride fractions. The evidence that diglycerides are actually the materials released from the fat body is as follows. When a digitonin solution was added to the sterol and the diglyceride fractions, all the radioactivity of the fraction remained in the supernatant, indicating that cholesterol (the major pupal sterol) contained no label. In no instance did the diglyceride fraction contain more than 10 percent sterol. Thin-layer chromatography (*6*) of this supernatant, and subsequent counting of the labeled eluate from the chromatogram revealed that almost all of the radioactivity (recovery 87 percent) was confined to a spot having an R_F identical to standard dipalmitin. After saponification of the labeled diglyceride fraction, another spot was detected on the chromatogram with an R_F identical to palmitic acid, and almost all of the radioactivity of the diglyceride fraction was now at the site of this palmitic acid (recovery 90 to 95 percent). Lastly, C^{14} carboxyl-labeled dipalmitin was made in our laboratory by hydrolysis of labeled tripalmitin with pancreatic lipase, and was then chromatographed. This labeled dipalmitin was then mixed with labeled hemolymph lipid and again chromatographed. One distinct peak was noted that corresponded exactly to that which we have designated the diglyceride peak in previous experiments.

All these findings indicate that diglyceride is the material released from the fat body into the hemolymph. Thus, the possibility exists that in cecropia at least, long-chain fatty acids are transported from the fat body to other tissues in the form of diglycerides. In the adult cecropia moth, the flight muscles utilize fatty acids almost exclusively but store little neutral fat (*7*), and fatty acids must be transported to this extremely active tissue, also, perhaps, in the form of diglycerides. When experiments identical to those already described were conducted on fat body from adult male cecropia moths (Table 2), the results were similar. In this instance the release of diglyceride was much greater, possibly because the fat body of the adult is more active metabolically than the pupal fat body.

Our results agree in part with those of Tietz (*8*), who found that the fat body of the locust also releases neutral fat into the hemolymph. However, Tietz did not attempt to identify chemically the nature of this neutral fat, but intimated that it was triglyceride. In preliminary experiments with a species closely related to the locust, and using techniques similar to those already described, we have confirmed some of the results of Tietz. In *Melanoplus differentialis*, we found that neutral fat was released in the form of diglyceride, as in cecropia. Thus, in representatives of two distinct orders of insects (Lepidoptera and Orthoptera), diglyceride appears to be important in the transport of lipids; this suggests that the mechanism may be common to many or all insects. In mammals, the main components of the transport system for long-chain fatty acids are, besides proteins, most probably triglyceride and unesterified fatty acids (*9*). In fact, the major component of the neutral fat in mammalian plasma is triglyceride (*10*) as opposed to the diglyceride of the hemolymph in cecropia pupae.

We then sought to discover whether the diglycerides are present free in the hemolymph or are conjugated to other molecules. By use of paper electrophoresis and staining procedures, Siakotos (*11*) demonstrated that several hemolymph proteins are conjugated to lipids in the cockroach (*Periplaneta americana*). On the basis of staining with sudan black, he suggests that at least two of these proteins carry neutral lipid and that this is the means by which lipid is transported in the roach. By a similar staining procedure and the use of polyacrylamide-gel electrophoresis (*12*), we were able to show that

Table 2. Release of glycerides and unesterified fatty acid from pupal fat body previously labeled with palmitic acid-1-C^{14}. Samples (300 mg) of labeled pupal fat body, containing 34,300 count/min of triglyceride, 133,800 count/min of diglyceride, and 37,300 count/min of unesterified fatty acid, were incubated with 1 ml of phosphate saline containing 1 μmole of glutathione; with 1 ml of 3.5 percent crystalline bovine serum albumin containing 1 μmole of glutathione; with 3.5 percent egg albumin in saline containing 1 μmole of glutathione; or with 1 ml of saline containing 0.25 ml of cell-free hemolymph and 1 μmole of glutathione, for 60 minutes. Samples (200 mg) of labeled adult fat body, containing 64,575 count/min of triglyceride, 253,900 count/min of diglyceride, and 15,580 count/min of unesterified fatty acid were incubated under the same conditions.

Incubation medium	Release in medium					
	Triglyceride		Diglyceride		Unesterified fatty acid	
	Count/min	Released (%)	Count/min	Released (%)	Count/min	Released (%)
			Pupal fat body			
Pupal hemolymph-saline	255	0.7	12,360	9.3	1,965	5.3
Serum albumin	185	0.5	400	0.3	4,080	10.9
Egg albumin	253	0.7	890	0.6		
Phosphate-saline	150	0.4	525	0.4	94	0.2
			Adult fat body			
Pupal hemolymph-saline	527	0.8	52,665	21.0	927	5.9
Phosphate saline	439	0.7	2,150	0.8	465	3.1

there are at least two distinct hemolymph proteins in cecropia pupae, which are lipoproteins. By cutting these bands from the gels and refluxing them in an ethanol : ether mixture to release the lipid moiety, we could demonstrate that a major portion of the radioactive diglyceride in the hemolymph is situated in these bands. This suggests that lipoprotein complexes with diglyceride as the lipid moiety, are the means by which cecropia, and perhaps other insects, transport lipids. This would be analogous to the situation in mammals where triglycerides and unesterified fatty acids are probably transported in combination with plasma proteins.

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15 October 1963

Swamp Mosquito, Culiseta melanura: Occurrence in an Urban Habitat

Abstract. *Adult* Culiseta melanura *were collected in Boston during three consecutive years (1961–63). Breeding sites were found along the bank of an impounded stream and in a small water collection in a concrete-lined pit.*

The reported restriction of *Culiseta melanura* (Coq.) to fresh-water swamps (*1, 2*) is significant because of the apparent potential of this mosquito as a vector of eastern equine encephalitis. Heretofore it has not been regarded as an important vector (*3*) because it has not been found near centers of human population. However, during the course of studying mosquito populations in Boston, Massachusetts, numerous *C. melanura* adults were captured.

The adults were collected in a dormitory of the Harvard Medical School, in the immediate vicinity of which are many institutional buildings. A small impounded stream, the Muddy River, is located about 450 meters away. Although its banks are cleaned periodically, eroded pockets and rodent holes are present along its margins. The nearest fresh-water swamp is at least 11 kilometers distant.

Adult mosquitoes (*C. melanura, C. minnesotae* Barr, *Culex restuans* Theobald, *C. salinarius* Coq., and *C. pipiens* L.) were collected at several sites, but *C. melanura* was found only in a utility tunnel beneath the building near an open window. A 1.8 × 0.9 m air shaft, containing approximately 10 cm of water rose 6 m from the window to the street. Mosquito larvae were generally present in this water, in a nearby catch basin, and in a variety of temporary water collections. Collections of resting adult mosquitoes were made at least once each week for 4 years. Samples of larvae were also taken weekly from the air shaft and catch basin, and, less regularly, from the other sites. Specimens were identified, and, when possible, succeeding stages were reared in the laboratory. Thus, identification of wild-caught females was frequently supplemented by study of larvae and males.

Sixteen adult *C. melanura* were collected in the utility tunnel during the course of the study (Table 1). Their abundance appeared to vary from year to year, but, in general, they were collected singly during the warm months. The capture of males and of gravid females suggested that a breeding source was nearby.

A systematic search for *C. melanura* larvae was conducted during the 15-month period ending October 1963 (*4*). Although more than 3000 larvae were captured (*Culex pipiens, restuans,* and *territans* Walker), no *C. melanura* were found until the end of August 1963. At this time, two egg rafts, which gave rise to *C. melanura* larvae, were collected from a pool that had previously been sampled 11 times. This permanent site (0.9 m × 1.2 m × 10 cm) was in an eroded portion of the bank of the Muddy River. The pool was sheltered by thick brush and, where the eggs were found, had undercut the bank. No larvae of this species were found in the pool in subsequent collections. One month later, however, 43 *C. melanura* larvae were discovered in the breeding site in the air shaft.

In the study area *C. melanura* were apparently well adapted to the man-made environment in that they were present during each of several years. The absence of prior records concerning these mosquitoes in urban areas may be due to their apparently elusive habits. Adults were recovered from the deep underground site but not in one at street level; the larvae seemed unusually sensitive to disturbance, and because they remained submerged for long periods, were difficult to collect. These difficulties are compounded by the morphologic similarities between *C. melanura* and *Culex pipiens,* which may result in *Culiseta melanura* being overlooked when *Culex pipiens* is abundant.

The breeding of *Culiseta melanura* appeared to be discontinuous, and adult abundance seemed to vary from time to time. Indeed, the distribution of this species is usually described as being focal (*1, 2, 5*). Nevertheless, it is conceivable that relatively dense populations of this mosquito could develop in the vicinity of human habitations under favorable conditions.

The possible role of *C. melanura* as

Table 1. Adult *Culiseta melanura* collected during 4 years, in a utility tunnel.

Date	Sex	Ovarian state
	1960	
—	—	—
	1961	
4 Sept.	F	Resting
16 Sept.	F	Resting
	1962	
18 June	F	Gravid
14 July	F	Gravid
31 July	F	Resting
6 Aug.	F	Resting
23 Aug.	F	Resting
24 Aug.	F	Gravid
29 Aug.	F	Gravid
11 Sept.	M	
	1963	
15 Aug.	F	Resting
27 Aug.	F	Resting
6 Sept.	F	Resting
1 Oct.	F	Resting
1 Oct.	F	Resting
1 Oct.	M	