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Lipid synthesis by ovaries and fat body of *Aedes aegypti* (Diptera: Culicidae)*

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Abstract. Synthesis of fatty acids by the ovaries of *Aedes aegypti* was demonstrated by incubating ovaries in tritiated water. Mostly phospholipids were synthesized, however, in mature oocytes the vast majority of lipids are triacylglycerol. 80% of the fatty acids synthesized in vitro were saturated while in the mature oocytes 70% of the fatty acids are unsaturated. These results suggest that lipids synthesized in the oocytes contribute only a minor portion of the lipid complement of the egg and that most of the oocyte lipids are taken up from external sources. The amount of lipid synthesized in vitro by the ovaries was extremely small, less than 1/1000 of the amount of lipid that accumulates in the oocytes. This result, however, may have been influenced by the in vitro conditions. Fat body, under the same conditions, synthesized less than 1/100 of the amount synthesized in vivo. The rate of lipid synthesis in this in vitro system was low, however, the regulation of lipid synthesis appeared to function. Lipid synthesis in ovaries and fat body was strongly inhibited by *Tabanus* adipokinetic hormone, whereas four other peptides isolated from *A. aegypti* did not influence lipid synthesis. In the fat body of sugar-fed mosquitoes, lipid synthesis was twice as great as in blood-fed animals.

INTRODUCTION

Oviparous animals must package all the reserves needed by the developing embryo into their eggs. This is well acknowledged in the case of proteins. The major egg protein, vitellin, as well as its hemolymph precursor, vitellogenin, have been isolated and characterized in many animals including insects (Kunkel & Nordin, 1985). The regulation of the genes for vitellogenins has been examined (Wyatt, 1991), and other egg proteins have been characterized (Law, 1990). Eggs, however, contain other substances as well, especially lipids and carbohydrates, which have received little attention. Lipids are needed for the formation of cell membranes when the single cell of an egg develops into a multicellular embryo. A developing embryo also requires large amounts of energy and, for any organism, the most efficient way to store energy is in the form of lipids. In *Manduca sexta* eggs, about 12% of the fresh weight or 40% of the dry weight is reported to be lipid (Kawooya & Law, 1988). For the eggs of the yellow fever mosquito *Aedes aegypti*, from 35 to 65% lipid have been reported; the larger the female, the smaller is the percentage of lipids in the eggs (Briegel, 1980). Troy et al. (1975) reported that 35% of *A. aegypti* eggs is lipid. An egg raft of the mosquito *Culex quinquefasciatus* consists of approximately 250 μg protein, 92 μg lipid and 20 μg glycogen. It has been estimated that 90% of embryonic

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respiration is supported by lipid (Van Handel, 1993), using up approximately 45% of the total lipids of an egg.

There are two ways in which lipids can accumulate in oocytes; (a) the lipids can be synthesized in the oocytes, or (b) the lipids can be synthesized elsewhere and transported to and taken up by the oocyte. Very little is known about lipid synthesis by insect oocytes. In locusts it was demonstrated that oocytes can synthesize triacylglycerol (TG) from fatty acids and glycerol (Lubzens et al., 1981; Ferenz, 1985). These results, however, do not show that fatty acids can be synthesized. They only demonstrate that oocytes can use fatty acids to synthesize more complex lipids.

It would be very difficult if not impossible to determine in vivo whether lipids are synthesized in the oocytes, or are synthesized elsewhere and transported to the oocytes. This question is better addressed in vitro. Glucose or acetate are useful as radioactive precursors to demonstrate that a tissue can synthesize lipids, however, these precursors are not suited to estimate the amount of fatty acids synthesized. The tissue contains unknown amounts of these precursors and of the intermediates in the synthesis of fatty acids, thus diluting the radioactive precursor by an unknown factor. Furthermore, these precursors are not suitable if the synthetic capacities of different developmental stages are to be compared, since the amount of precursor in the tissue might vary strongly. Tritiated water is recommended for quantitative analysis of the amount of fatty acids synthesized (Lowenstein, 1972). During the synthesis of fatty acids, two hydrogen atoms from NADPH and two hydrogen atoms from water are incorporated for every two carbon atoms. Because the amount of water in which the tissue is incubated is known, a quantitative estimate of synthesized fatty acids is possible, as well as comparison of the synthetic capacity of different tissues. Fatty acid synthesis by *M. sexta* oocytes was measured using this method (Kawooya & Law, 1988), but no values regarding the amount of lipid synthesized were presented in that paper. By comparing the ratio of saturated to unsaturated fatty acids synthesized with the ratio found in the oocytes, it was estimated that less than 1% of the fatty acids found in eggs of *M. sexta* are synthesized by the oocytes.

In an attempt to understand the origin of lipids in eggs of the mosquito *A. aegypti*, we tested whether mosquito oocytes are able to synthesize fatty acids. In mosquitoes it is not possible to examine this in individual oocytes, therefore ovaries were used in this study.

MATERIAL AND METHODS

A. aegypti (NIH-Rockefeller strain) were reared at 27.5 ± 0.5°C, 80% relative humidity with a photoperiod of 16 : 8, using standard techniques (Baker et al., 1983). The mosquitoes had free access to sugar water on a cotton pad. 3–5 days after emergence, females had one bloodmeal on an anesthetized rabbit.

Ovaries were dissected in *Aedes* saline (Hagedorn et al., 1977) under a dissecting microscope. If not stated otherwise, the ovaries were dissected from females 24 to 26 h after a blood meal. Six pairs of ovaries were incubated in 100 µl of *Aedes* saline or *Aedes* medium (Bohm et al., 1978) containing 4 mCi of tritiated water. Incubations lasted 4 h if not otherwise indicated. After the incubation, the ovaries were washed in four changes of nonradioactive saline, and four groups of six pairs of ovaries were combined. Fat body preparations, i.e. abdomens free of gut and ovaries (Fallon et al., 1974), were incubated in groups of five in 100 µl, and two groups were combined. The tissues were homogenized in glass/glass homogenizers with chloroform : methanol (Folch et al., 1957). The homogenate was washed with several changes of methanol : water until the aqueous phase contained only a few hundred dpm.

The organic phase was well dried under nitrogen. The dried lipids were taken up in 100 µl of chloroform, and 20 µl of this solution were applied to a thin layer silica gel plate (Si250, Baker, Phillipsburg,

NY). Lipid classes were separated using a solvent system consisting of hexane : ether : acetic acid (60 : 40 : 1). Lipids were visualized in iodine vapor and identified by standards. Spots were marked and, after evaporation of the iodine, scratched out and the radioactivity was determined.

For further analysis, fatty acids were transesterified to their methyl esters. Transesterification was performed by transferring the remaining 80 µl of the chloroform solution to a round bottom tube. It was dried and incubated with 0.5 ml of 5% KOH in methanol for 1 h at 60°C. Then, 0.5 ml of 14% boron trifluoride in methanol was added and this mixture was incubated for 1 h at room temperature. The reaction was stopped by the addition of 2 ml of water, and methyl esters of fatty acids were extracted by washing 6 times with 2 ml of hexane. The resulting samples were dried with a gentle stream of nitrogen. The walls of the tube were rinsed with more hexane to concentrate the sample within the tip of the tube and the sample was dried again. The methyl esters were dissolved in 100 µl of hexane and applied to a thin layer silica gel plate impregnated with $AgNO_3$. The plate was developed in benzene : ether (90 : 10). Methyl esters were visualized under ultraviolet light after spraying with 0.02% 2',7'-dichlorofluorescein in methanol (Shibahara et al., 1986).

Recovery of lipids was tested by adding a known amount of radioactive triacylglycerol to ovaries which were extracted by the procedure described above. The radioactivity of the methyl esters of fatty acids was determined. Recovery was between 75 and 85%.

Peptides: *Aedes* head peptide 1 and 3, *Aedes* allatotropin (Veenstra, in prep.) and *Aedes* leucokinin (Veenstra, 1994) were generous gifts by J.A.Veenstra (University of Bordeaux I, France). *Tabanus atratus* (Tabanidae) adipokinetic hormone (AKH) was purchased from Penninsula Laboratories (Belmont, CA). The peptides were tested by adding 100 pmol to 100 µl of incubation medium. The tabanid AKH was also tested at 10 pmol.

Gas chromatography: Fatty acid methyl esters from lipid extracts were separated by gas chromatography with a Shimadzu GC-mini 1. The column used was a 10% Silar-10C, mesh 100/120. The starting temperature was 140°C which was increased to 240°C (4°C per minute).

RESULTS

OVARIES: Ovaries obtained from females 24 to 26 h after a blood meal were incubated in *Aedes* saline with tritiated water. The ovaries synthesized fatty acids and the amount synthesized increased for at least 4 h. One pair of ovaries incorporated 74 dpm into lipids in 1 h. Of the lipids synthesized, 26% were triacylglycerol (TG) and 56% phospholipid (PL) (Table 1). Transmethylation of fatty acids demonstrated that 15 dpm of the 74 dpm were incorporated into fatty acids (Table 2). The remaining radioactivity was detected in the aqueous phase, most likely in the form of glycerol. When glycerol phosphate is formed from dihydroxyacetone phosphate, hydrogen from water is incorporated. In addition, the hydroxyl groups of glycerol can exchange hydrogen with water. Because the 4 mCi of tritiated water was highly diluted in a total of 100 µl of water which is 5.5 mmol, the 15 dpm in fatty acids correspond to 17 ng of fatty acids synthesized in 2 days. Lipids do not consist of fatty acids only. TG weighs about 5% more than its fatty acids and PL weighs about 30% more. Therefore, the total weight of lipid synthesized is somewhat higher than the weight of the free fatty acids synthesized. The amount of lipids synthesized is very small. This could be a result of limitations of the in vitro system rather than the synthetic capacity of the ovaries. When the ovaries were incubated in *Aedes* medium instead of saline, no change in the amount of fatty acids synthesized was observed. Enriching the saline or the medium with oxygen at the beginning of the experiment did not change the results either. Addition of 25 mM glucose to *Aedes* saline doubled the amount of fatty acids synthesized.

TABLE 1. Lipid classes synthesized in vitro by ovaries and fat body and lipid classes found in mature oocytes (% of total lipids). PL – phospholipid, MG – monoacylgylcerol, DG – diacylglycerol, FFA – free fatty acids, TG – triacylglycerol, HC – hydrocarbon and cholesterol ester.

		PL	MG	DG	FFA	TG	HC	n
Synthesis	Ovaries (24 h after a blood meal)	55.5±4.2	3.6±1.7	8.8±1.1	4.8±2.6	25.8±3.3	2.1±0.4	8
	Fat body (sugar-fed)	11.8±1.3	0.3±0.2	8.1±0.7	2.4±0.9	71.0±1.9	6.2±1.4	15
	Fat body (24 h after a blood meal)	18.2±1.7	0.3±0.2	8.8±2.9	3.0±0.3	69.7±1.9	1.9±0.9	9
Content	Ovaries (48 h after a blood meal)	16.8±1.6	Traces	8.3±2.3	Traces	66.0±3.5	8.8±2.4	4

TABLE 2. DPM incorporated into fatty acids per tissue in 1 h; a is significantly different from b and c from d at $P < 5\%$ (Student t-test).

		Average	Maximum	n
Ovary		15.3 ± 3.2	30	8
Fat body	Sugar-fed	220.6 ± 44.5	562.6	14^{a}
	Sugar-fed plus AKH	68.3 ± 13.4	135.9	9^{b}
	Blood-fed	99.2 ± 19.1	171	$9^{b,c}$
	Blood-fed plus AKH	45.6 ± 9.6	82.3	7^{d}

The ratio of saturated to unsaturated fatty acids was also determined: 82% of the synthesized fatty acids where saturated (Table 3). In the oocytes, as well as in the fat body and in *Aedes* lipophorin, only about 30% of the fatty acids were saturated (Fig. 1).

TABLE 3. Fatty acid composition of total egg lipids and of egg lipids synthesized in vitro.

	Total fatty acids from oocytes, two days after a blood meal (%) n = 4	Fatty acids synthesized by ovaries in vitro, 24 h after a blood meal (%) n = 6
Saturated fatty acids	32 ± 0.4	82.4 ± 3.8
Unsaturated fatty acids	67.9 ± 0.6	17.6 ± 3.8

FAT BODY: Fat body reparations from sugar-fed females synthesized much more lipid than ovaries. One fat body preparation incorporated 220 dpm into fatty acids in 1 h (Table 2). This corresponds to about 242 ng fatty acids synthesized in two days, which is much smaller than the amount of lipid synthesized in vivo by the fat body of females feeding on sugar water. Sugar-fed females increase the lipid content of their fat body by about 200 µg during the first 5 days after emergence (Ziegler & Ibrahim, unpublished). The percentages of the lipid classes synthesized, however, are very close to the percentages found in fat body (PL 12% and TG 71%) (Table 1). In addition, the regulation of fatty acid synthesis in fat body appears to be intact in this in vitro system. When fat bodies from blood-fed females were tested, the synthesis of fatty acids was significantly ($P < 5\%$) reduced compared to sugar-fed females (Table 2).

INFLUENCE OF PEPTIDES: Peptides isolated from *Aedes* and *Tabanus* were tested to determine their influence on fatty acid synthesis. The *Aedes* head peptides 1 and 3, *Aedes*

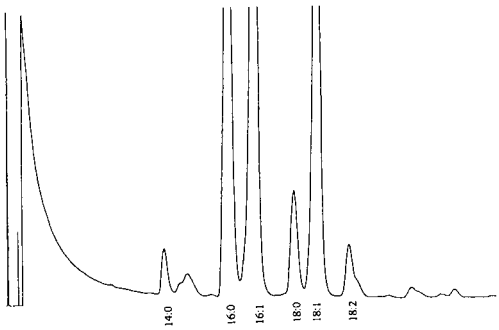

Fig. 1. Fatty acid composition of lipids from ovaries of females 24 h after a blood meal.

allatotropin, and *Aedes* leucokinin did not influence fatty acid synthesis. The addition of *T. atratus* AKH, however, strongly reduced the synthesis of fatty acids in fat body (Table 2). AKH was tested by adding either 100 pmol or 10 pmol. Both amounts used gave the same result, indicating that 100 pmol is an excess amount. In one experiment with ovaries, AKH at the dosage of 100 pmol was found to inhibit lipid synthesis in ovaries by more than 60%.

DISCUSSION

During the first 48 h after a blood meal, ovaries of *A. aegypti* acquire large amounts of lipids (Troy et al., 1975). In our colony, one pair of ovaries acquired 65 µg of lipids. 24 h after a blood meal ovaries had already accumulated about 30 µg (Ziegler & Lyons, unpublished). When ovaries of *A. aegypti* 24 to 26 h after a blood meal were incubated in tritiated water they clearly synthesized fatty acids, as tritium was incorporated into fatty acids. The incorporation increased for at least 4 h, thus an incubation time of 4 h was chosen. Most of the label was found in phospholipids and very little in triacylglycerol. The mature oocytes, however, contained mostly triacylglycerol and little phospholipid, which indicates that only a small amount of the lipids found in the eggs is synthesized in the ovary. This is supported by the fact that ovaries synthesize mostly saturated fatty acids while the oocytes contain mostly unsaturated fatty acids.

The amount of lipid synthesized by the ovary in vitro is very small; in two days one pair of ovaries synthesized in vitro about 20 ng, which is less than 1/1000 of the 60 µg of lipids the ovaries acquire in two days. If the ovaries in an in vitro system were as efficient in synthesizing lipids as the ovaries in an intact mosquito, this would clearly show that most of the egg lipids are synthesized outside the ovaries. However, we have reasons to doubt that the in vitro system is very efficient (see below). Nevertheless, these results taken together strongly suggest that only a minor fraction of the oocyte lipid complement is synthesized in the oocytes. In addition, preliminary results (Ziegler & Lyons, unpublished) indicate that ovaries incubated with mosquito lipophorin take up large amounts of lipids, which could account for the lipids in the eggs. This is in agreement with results in other insects including *M. sexta* (Kawooya & Law, 1988; Kawooya et al., 1988) and *Hyalophora cecropia* (Telfer et al., 1991).

During the first five days of adult life, the lipid content of fat bodies of sugar-fed *A. aegypti* increases from less than 100 µg to nearly 300 µg (Ziegler & Ibrahim, unpublished). This is similar to results in *Aedes sollicitans* (Van Handel, 1965). If fat bodies of sugar-fed females of *A. aegypti* are incubated in *Aedes* saline, they synthesize about 250 ng of lipid per fat body in 2 days. This is more than ten times the amount synthesized by the ovaries of the same animals, but is less than 1/100 the amount synthesized in intact mosquitoes. Fat bodies synthesized different lipid classes in vitro in the same ratios as those found in

total lipids extracted from fat bodies. In contrast to ovaries, fat bodies synthesized in vitro mostly TG. Guts were also tested for lipid synthesis, however, no incorporation of tritium into fatty acids was observed.

In attempts to increase the amount of lipids synthesized in vitro, different incubation conditions were tested. Incubations in *Aedes* medium instead of *Aedes* saline did not increase the amount of lipids synthesized. When the saline or the medium was enriched with oxygen before the incubations, the amount of lipids synthesized was not increased. Insect fat bodies are notorious for having a lower synthetic capacity in vitro than in vivo. The addition of 25 mM glucose to the saline doubled the synthesis of fatty acids. This value is still far below the synthesis one would expect from the in vivo results. At present, it is not possible to determine whether fat bodies incubated with glucose synthesize lipids more like in vitro, or whether the fat bodies are under glucose stress when that much glucose is added.

Fatty acids are synthesized in vitro by ovaries and fat bodies. The rate of synthesis in vitro is lower, at least for fat bodies, than it is in intact animals, however, the tissue appears to respond to factors controlling lipid synthesis. Sugar-fed female mosquitoes accumulate lipids in the fat body and this study demonstrates that lipid is synthesized by the fat body and not by the gut. The lipid content of fat bodies from sugar-fed females increases most markedly around the third day of adult life (Ziegler & Ibrahim, unpublished), indicating a maximal synthetic capacity around this day. In a preliminary experiment, the synthetic capacity of fat bodies from sugar-fed females of different ages was compared. Fat bodies from females on their third day of adult life showed by far the highest rate of synthesis. Fat bodies from blood-fed females synthesized only 50% of the amount synthesized by fat bodies from sugar-fed females. It appears that blood feeding shifts fat body metabolism from lipid synthesis to the transfer of lipids from fat body to oocytes.

Little is known about the regulation of lipid synthesis in insects. There are reports that the medial neurosecretory cells of the brain stimulate lipid synthesis in mosquitoes by an unknown factor (Van Handel & Lea, 1965). J.A. Veenstra has recently isolated several peptides from *A. aegypti* and made them available for testing. None of these peptides had any influence on lipid synthesis by the mosquito fat body. Adipokinetic hormone has been reported to inhibit lipid synthesis in insects. This was first shown in *Schistocerca gregaria* (Orthoptera) (Gokuldas et al., 1988). AKH of *A. aegypti* was not available; therefore, AKH of another dipteran, *T. atratus*, was added to the incubation medium. This AKH inhibited lipid synthesis in ovaries from blood-fed females as well as in fat bodies from sugar-fed females by 50 to 70%. AKH-treated fat bodies from blood-fed females synthesized only 20% of the amount of lipid synthesized by untreated fat bodies from sugar-fed females. The role of AKH in mosquitoes is presently unknown. In the blowfly *Calliphora erythrocephala* (Calliphoridae), there are indications that an AKH controls carbohydrate mobilization during flight (Vejbjerg & Normann, 1974) and AKH could have a similar function in mosquitoes. AKH, which is secreted during flight, appears to inhibit synthetic activities in insects; it is known to inhibit the synthesis of lipid (Gokuldas et al., 1988), RNA (Kodrík & Goldsworthy, 1995) and protein (Carlisle & Loughton, 1986), probably to save energy for flight, which is extremely energy demanding (Ziegler, 1985).

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