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Fat synthesis in cell-free preparations of the locust fat-body

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SUMMARY

It was shown that cell-free preparations of the fat-body of the migratory locust, *Locusta migratoria*, incorporated acetate into fatty acids in the presence of ATP, CoA, glutathione, Mg^{++}, TPN, malonate, α-ketoglutarate, and $KHCO_3$. The major fatty acid component synthesized was palmitic acid. The newly synthesized acids were esterified by the system with glycerol as glycerides and phospholipids. Mitochondria were not required for synthesis. Fat-body homogenates could also activate and decarboxylate malonate and form malonic acid by CO_2 fixation.

Fat metabolism in higher animals has been extensively studied for many years, and several of the intermediate steps of fat synthesis and degradation have been elucidated (1). Relatively little is known about fat metabolism in insects. Most insects have a fat-body which combines some of the functions of liver and adipose tissue of higher animals (2). As experimental specimen, the migratory locust, *Locusta migratoria*, was chosen. Weis-Fogh (3) had previously shown that during flight locusts utilized fat stored in the fat-body. From differences between the RQ values found in resting insects and during sustained flight, he calculated that 85% of the total energy spent was supplied by the oxidation of lipids. Recently it was shown by Meyer *et al.* (4) that a particulate fraction from the flight muscles of the desert locust could completely oxidize fatty acids *in vitro* under suitable conditions. Since locusts renew their fat reserves during rest and feeding, it was to be expected that the fat-body would synthesize lipids from suitable precursors. Clements (5) showed, indeed, that intact sheets of previsceral fat-body converted, *in vitro*, acetate, glucose, and amino-acids into fat. To study the mechanism of these reactions, cell-free extracts of fat-body tissue were prepared, and the incorporation of acetate into fatty acids was investigated. A preliminary account of this work has been presented before the Israel Chemical Society (6).

METHODS

Preparation of Cell-free Systems. Locusts (*Locusta migratoria*) were bred in glass cages and fed grass and oatmeal. The cages were kept at 28° and 70% humidity. Fat-body tissue was removed from male and female locusts 8 to 15 days after the last molt. The tissue obtained from a single insect was homogenized with 0.6 ml of a buffer containing 0.085 M K_2HPO_4, 0.009 M KH_2PO_4, 0.010 M $KHCO_3$, and 0.002 M GSH.[1] The homogenate was centrifuged at 400 × *g* for 5 minutes and then filtered through a small pad of cotton to remove fat which accumulated on top. To obtain subcellular particles, the homogenate was further centrifuged at 20,000 × *g* for 20 minutes. The supernatant was decanted, filtered to remove fat, and used without further manipulations. The precipitate was suspended in fresh buffer by homogenization. Usually a concentrated suspension of particles was prepared.

Enzymatic Assays. The method for assay of fatty acid synthesis was carried out as described by Wakil *et al.* (7). Acetate-1-C^{14} was incubated with cofactors and enzyme for 2 hours at 30° under air. The reaction was stopped by addition of 10% ethanolic KOH and the mixture was saponified for 3 hours. Following acidification, the long-chain fatty acids were extracted into petroleum ether (b.p. 40°–60°). Aliquots were taken from the extract for plating and counting. In some experiments when esterified lipids were isolated, the reaction was stopped by addition of 10 volumes ethanol-ether (3/1, v/v) followed by heating to the boiling point of the mixture.

To measure the decarboxylation of malonate-1-C^{14} and malonate-2-C^{14}, incubations were carried out in

[1] Abbreviations: GSH = glutathione; ATP = adenosine triphosphate; CoA = coenzyme A; DPN and TPN = diphospho- and triphosphopyridine nucleotides, respectively.

Thunberg tubes. The reaction was stopped by addition of 0.2 ml 5 N H_2SO_4 from the side bulb. After 10 minutes the tubes were evacuated. To trap liberated $C^{14}O_2$, the air was passed through a 10% NaOH solution. Na_2CO_3 was converted to $BaCO_3$ for plating and counting.

$C^{14}O_2$ fixation was measured as described by Tietz and Ochoa (8). To identify the labeled acids formed, the reaction was stopped by addition of H_2SO_4 to a final concentration of 3N. The protein-free supernatant was mixed with Celite® (9) and the acids extracted with ether in a Soxhlet apparatus for 3 hours.

To measure acetate and malonate activation, the substrates were incubated with cofactors, hydroxylamine, and enzyme. Formation of acethydroxamic and malonomonohydroxamic acid was estimated by forming the colored iron complex (10). To isolate the hydroxamates formed, the reaction was stopped by addition of 10 volumes of ethanol. The protein-free supernatant was evaporated under reduced pressure and the residue extracted into a small volume of ethanol.

Separation of Lipids. Phospholipids were separated from a mixture of lipids by precipitation with acetone (11) after addition of carrier yolk-phospholipids. To isolate cholesterol, the acetone supernatant was extracted with petroleum ether after saponification and cholesterol precipitated from the extract with digitonine (12) after addition of carrier cholesterol. Neutral-fat fatty acids were extracted from the saponified mixture after acidification.

After it had been shown that only negligible amounts of acetate were incorporated into unsaponifiable matter, the acetone supernatant (after precipitation of phospholipids) was passed through a MgO-Celite® column (11) and glycerides and free fatty acids were eluted with acetone and methanol, respectively. Free fatty acids were obtained from the purified phospholipids and glycerides after saponification.

Paper Chromatographic Procedures. Long-chain fatty acids were resolved according to Buchanan (13) with acetic acid-88% formic acid-30% H_2O_2 (6/1/1, v/v) as developing solvent. Chromatograms were run for 18 hours at 30°.

Acetic and malonic acids were separated according to Isherwood and Hanes (14), with propanol-30% ammonia (6/4, v/v) as developing solvent.

Acethydroxamic and malonomonohydroxamic acids were separated according to Fink and Fink (15) with butanol-acetic acid or water-saturated phenol, and identified by spraying with ethanolic $FeCl_3$ (16).

C^{14} Assays. Lipid material was plated on planchets which were lined with lens paper. Not more than 1 mg material per cm^2 was plated to avoid self-absorption corrections. $BaCO_3$ and cholesterol digitonide were pipetted directly onto the planchets and dried under an infrared lamp. Self-absorption corrections were made when necessary. To locate C^{14}-labeled material on a chromatogram, the paper strip was cut into 1 cm pieces and each piece was counted separately. An end window GM-counter was used throughout.

MATERIALS

The following substances were obtained from commercial sources: crystalline ATP, DPN, and TPN (Sigma Chemical Company); CoA (Pabst Laboratories). Acetate-1-C^{14}, $BaC^{14}O_3$, ethylmalonate-1-C^{14}, and ethylmalonate-2-C^{14} were obtained from the Radiochemical Centre, Amersham, Bucks, England. Ethylmalonate was saponified before use. Since the malonate-1-C^{14} sample contained 2% contamination and the malonate-2-C^{14} 50% contamination, the acids were purified by chromatography on Whatman No. 3MM. paper (14).

RESULTS

Conditions for Fatty Acid Synthesis by Fat-Body Homogenates and Extracts. To determine optimal conditions for fatty acid synthesis from acetate by fat-body homogenates, the latter were incubated with acetate-1-C^{14} in the presence of different substrates and cofactors, and the recovery of C^{14} in fatty acids was estimated. The results are summarized in Table 1.

TABLE 1. COMPONENTS REQUIRED FOR FATTY ACID SYNTHESIS

Cofactor Omitted	Acetate Incorporated into Fatty Acid
	μmoles
None*	0.33
ATP	0.05
CoA	0.14
GSH	0.17
DPN	0.33
TPN	0.19
$MgCl_2$	0.01
$MnSO_4$	0.32
Malonate	0.01
α-ketoglutarate	0.20
$KHCO_3$	0.08

* The complete system contained (in μmoles): acetate 5 (80,000 cpm acetate-1-C^{14}), ATP 5, CoA 0.1 mg, GSH 5, DPN 0.5, TPN 0.5, $MgCl_2$ 10, $MnSO_4$ 0.5, malonate 20, α-ketoglutarate 10, $KHCO_3$ 10, and 0.6 ml (2 mg protein) homogenate in a total volume of 1 ml. The pH was adjusted to 7.0 by addition of HCl.

ATP, $MgCl_2$, $KHCO_3$, and malonate were required for synthesis; CoA, GSH, and TPN further stimulated the system; DPN and $MnSO_4$ had no effect. Malonate could not be replaced by any intermediate of the glycolytic or Krebs cycle. However, addition of some of these intermediates in the presence of malonate caused further stimulation. The best results were obtained with α-ketoglutarate. Citrate and isocitrate had no effect; glucose-6-phosphate showed some stimulation (Table 2).

TABLE 2. REPLACEMENT OF MALONATE BY OTHER SUBSTRATES

Additions	Acetate Incorporated into Fatty Acids
	μmoles
None*	0.01
α-ketoglutarate	0.09
Citrate	0.06
Malonate	0.25
Malonate + α-ketoglutarate	0.40
Malonate + citrate	0.25
Malonate + G6P	0.33
Malonate + α-glycerophosphate	0.27

* The complete system contained acetate and cofactors as mentioned in the footnote to Table 1. The following substances were added when indicated: α-ketoglutarate, citrate, glucose-6-phosphate, and α-glycerophosphate, 10 μmoles each; malonate 20 μmoles, and 0.6 ml. homogenate (3 mg protein) in a total volume of 1 ml.

The optimal concentrations of the essential components of the system were as follows: ATP 2.5 to 10 μmoles, CoA 0.1 mg or more, GSH 5 μmoles or more, TPN 0.5 μmole, $MgCl_2$ 5 to 10 μmoles, $KHCO_3$ 10 to 20 μmoles, malonate 15 to 25 μmoles, and α-ketoglutarate 10 μmoles. The optimal pH was 7.0. Whereas at pH 7.0, 0.44 μmole of acetate was incorporated into fatty acids, at pH 6.5 only 0.20 μmole, and at pH 7.8, 0.25 μmole of acetate was used.

Particles were not required for fatty acid synthesis. Under optimal conditions the particle-free supernatant was as active as the original homogenate in incorporating C^{14}-acetate into fatty acids.

Isolation of Lipid Components Synthesized by Fat-Body Homogenates and Extracts. To determine the lipid components which were synthesized by the fat-body preparations, phospholipids, glycerides, free fatty acids, and unsaponifiable matter were isolated as described under Methods. As can be seen from Table 3, only negligible amounts of C^{14} were recovered in

TABLE 3. C^{14} CONTENT OF ISOLATED LIPID COMPONENTS

Lipid Fraction	C^{14} Recovered in Fraction	
	Homogenate	Supernatant
	cpm × 10^3	*cpm* × 10^3
Lipid mixture (total)	127	121
Phospholipids	23.3	11.6
Glycerides	102	108.5
Unsaponifiable matter	.625	
Cholesterol digitonide	.045	
Free fatty acids	.950	

Incubation mixture as described in the footnote to Table 1. 800,000 cpm acetate-1-C^{14} were added per tube. Two tubes were pooled before the component lipids were separated.

unsaponifiable matter and the cholesterol digitonide precipitated from it; C^{14}-acetate was incorporated only into fatty acids. The newly synthesized fatty acids were esterified with glycerol and did not accumulate as free acids. The fat-body homogenate esterified 18% as phospholipids and 81% as glycerides; the mitochondria-free supernatant, 10% as phospholipids and 90% as glycerides.

To determine the composition of the newly synthesized fatty acids which were contained in the phospholipid and glyceride fractions, the acids were isolated and an aliquot was separated by paper chromatography. With the highly labeled glyceride-fatty acids a clear separation of the acids was obtained (Fig. 1).

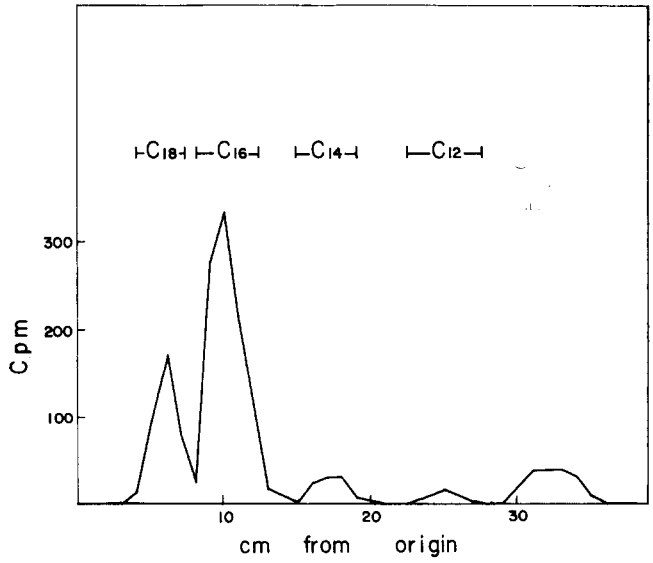

FIG. 1. Separation by paper chromatography of glyceride-fatty acids synthesized by locust homogenate.

The fatty acids of the phospholipid fraction did not contain enough C^{14} to obtain a significant distribution of the counts when a small amount of material was used, as required by the paper chromatographic method. As can be seen from Table 4, C^{14} was found in all *n*

TABLE 4. COMPOSITION OF GLYCERIDE-FATTY ACIDS

Fatty Acid	C^{14} Recovered in Fractions as Per Cent of Total	
	Homogenate	Supernatant
Stearic (octadecanoic)	22	2.5
Palmitic (hexadecanoic)	60	55
Myristic (tetradecanoic)	5	18
Lauric (dodecanoic)	2	3.5
Shorter and unsaturated acids	11	21.0

long-chain fatty acids with an even number of carbon atoms from 12 to 18. The major fatty acid component was palmitic acid. A considerable amount of radioactivity traveled with the fastest peak representing fatty acids which contain less than 12 carbon atoms and unsaturated acids. Except for minor differences, the pattern of labeling in the glyceride-fatty acids obtained from homogenate and particle-free supernatant was similar.

Activation of Acetate and Malonate. The presence of activating enzymes for acetate and for malonate in locust fat-body preparations could be readily shown when these substrates were incubated in the presence of cofactors, hydroxylamine, and enzyme. In the particle-free supernatant, hydroxamic acid formation occurred in the absence of added substrates. This activation was retained even when the supernatant was first dialyzed overnight. Addition of acetate stimulated hydroxamic acid formation only slightly. Malonate was not activated by this system (Table 5). The formation of hydroxamic acid was dependent on the addition of ATP and CoA. The hydroxamic acids formed by the supernatant were separated by chromatography on paper. In the absence of added substrates two spots were identified: one corresponded to acethydroxamic acid, the second moved faster and corresponded to hydroxamic acids of longer chain length. In the presence of acetate the acethydroxamic acid spot was augmented. When the particles were tested for activating enzymes, addition of malonate, but not of acetate, resulted in the formation of hydroxamic acid.

TABLE 5. FORMATION OF HYDROXAMIC ACIDS

Additions*	Hydroxamic Acid Formed	
	Supernatant	Particles
	μmoles†	*μmoles*†
None	0.87	0.02
Acetate	1.06	0.02
Malonate	0.85	0.68

* The reaction mixture contained (in μmoles): hydroxylamine low in salt content, adjusted to pH 8.0 500, ATP 10, $MgCl_2$ 10, CoA 0.1 mg, GSH 5 and 0.5 ml of enzyme (2.5 mg protein) in a total volume of 1 ml; 20 μmoles of acetate or malonate were added as indicated. Incubated 1 hour at 30°.

† Results expressed as μmoles of acethydroxamic acid.

The hydroxamic acid formed was separated by chromatography and identified as malonomonohydroxamic acid.

Carbon Dioxide Fixation. To study the effect of CO_2 on fatty acid synthesis, $C^{14}O_2$ fixation by fat-body homogenates was investigated. $C^{14}O_2$ was readily fixed. Addition of acetate stimulated the fixation only very slightly; propionate was slightly inhibitory (Table 6).

TABLE 6. FIXATION OF $C^{14}O_2$ BY FAT-BODY HOMOGENATES

Additions*	CO_2 Fixed
	μmoles
None	0.34
Acetate	0.42
Propionate	0.26

* The reaction mixture contained (in μmoles): ATP 5, $MgCl_2$ 10, CoA 0.1 mg, GSH 5 and 0.6 ml homogenate (pH 7.8) in a total volume of 0.8 ml. Ten μmoles of acetate or propionate were added as indicated. Fourteen μmoles of $K_2C^{14}O_3$ containing 200,000 cpm in 0.2 ml were tipped in from the side bulb after 5 minutes' equilibration, and the incubation continued for 2 hours.

More $C^{14}O_2$ was fixed at pH 7.8 (0.31 μmole) than at pH 7.0 (0.22 μmole). Both mitochondria and supernatant were required for $C^{14}O_2$ fixation; each fraction alone was inactive. The $C^{14}O_2$ which had been fixed was not incorporated into fatty acids; it was found in ether-extractable acids. These were separated by chromatography. Of the counts, 91% were recovered in malonic acid, 9% in acetic acid.

Metabolism of Malonate. To study the effect of malonate on fatty acid synthesis, the metabolism of malonate by fat-body preparations was investigated. When malonate-1-C^{14} was added instead of acetate-1-

C^{14} to the reaction mixture, C^{14} was recovered in $BaCO_3$ and fatty acids. To test for a possible connection between the decarboxylation of malonate and fatty acid synthesis, the incorporation of acetate-1-C^{14} and of malonate-1-C^{14} into CO_2 and fatty acids by homogenates, supernatant, and particles was compared. As can be seen from Table 7, acetate was not oxidized under the experimental conditions, but it was readily incorporated into fatty acids by the homogenate and particle-free supernatant. Malonate-1-C^{14} was decarboxylated by the homogenate and particles; only trace activity was found in the supernatant. The homogenate also utilized malonate-1-C^{14} for fatty acid synthesis, but to a smaller extent than acetate. Although the incorporation of acetate-1-C^{14} by the supernatant was higher than by the homogenate, utilization of malonate by the supernatant was negligible. The particles converted some malonate into fatty acids, and the synthesis of fatty acids from malonate exceeded that from acetate. However, in all experiments more C^{14} was found in $BaCO_3$ than in fatty acids; decarboxylation seems to exceed the utilization of malonate for fatty acid synthesis. Addition of acetate to a reaction mixture which contained malonate-1-C^{14} did not affect the metabolism of malonate by fat-body preparations. These experiments were repeated with malonate-2-C^{14}. As expected, no C^{14} was recovered in $BaCO_3$. The pattern of C^{14} incorporation into fatty acids was similar to that obtained with malonate-1-C^{14}. However, the recovery of C^{14} was lower than expected and did not amount to twice the activity found with malonate-1-C^{14}.

TABLE 7. METABOLISM OF ACETATE-1-C^{14} AND OF MALONATE-1-C^{14}

Preparation	Additions*	C^{14} Incorporated into	
		$BaCO_3$	Fatty Acids
		μmoles	*μmoles*
Homogenate	Acetate-1-C^{14} + malonate	0.0	0.21
	Malonate-1-C^{14}	0.10	0.05
	Malonate-1-C^{14} + acetate	0.09	0.04
Supernatant	(1-C^{14}) acetate + malonate	0.0	0.31
	(1-C^{14}) malonate	0.01	0.02
	(1-C^{14}) malonate + acetate	0.01	0.01
Particles	(1-C^{14}) acetate	0.0	0.02
	(1-C^{14}) malonate	0.15	0.04
	(1-C^{14}) malonate + acetate	0.17	0.05

* Conditions of incubation as described in the footnote to Table 1; 210,000 cpm malonate-1-C^{14} added as indicated.

Decarboxylation of malonate by the particles was further studied. In the absence of ATP, decarboxylation dropped from 0.19 μmole to 0.07 μmole. A requirement for CoA could also be shown. The decarboxylation was faster at pH 7.8 than at pH 7.0; at pH 7.0 it dropped to 0.09 μmole.

DISCUSSION

It has been shown that the biosynthesis of fatty acids in cell-free preparations of locust fat-body required the addition of ATP, CoA, GSH, Mg^{++}, TPN, $KHCO_3$, malonate, and α-ketoglutarate. These requirements are very similar to those reported for liver (17) and mammary gland (18) extracts. A similar system was also obtained by Zebe and McShan (19) from the fat-body of the moth *Prodenia eridania*. The similarity in substrate and cofactor requirement of the different systems suggests that a similar mechanism for fatty acid synthesis is operative. CoA, ATP, and Mg^{++} are therefore required for acetyl-CoA formation. The requirements of malonate and α-ketoglutarate for fatty acid synthesis were described in mammary gland extracts (18), and it was suggested that α-ketoglutarate reduced added pyridine nucleotides. The effect of malonate could not be explained. The requirement of $KHCO_3$ for fatty acid synthesis in liver extracts was first described by Gibson *et al.* (20). In this system $KHCO_3$ acted catalytically. It was required for the formation of malonyl-CoA from acetyl-CoA (21). In the presence of TPNH, malonyl-CoA condensed with acetyl-CoA to yield fatty acyl-CoA (22); the newly fixed CO_2 was lost in the condensation reaction. To study the effects of malonate and $KHCO_3$ in the locust system, the C^{14}-labeled substrates were used. The presence of activating enzymes for acetate and malonate, a system which decarboxylated malonate and a system which fixed CO_2 and formed malonate, were found in the locust fat-body homogenate. There was, however, no correlation between the distribution of the fatty acid synthesizing system and the above-mentioned enzymes. Whereas acetate activation and fatty acid synthesis occurred in the particle-free supernatant, particles were required for the activation and decarboxylation of malonate. A particulate system from kidney, which activated and decarboxylated malonate, was previously described by Nakada *et al.* (23). CO_2 was fixed only in the presence of both particles and supernatant. Since activation of acetate occurred in

the supernatant, it seems likely that the mitochondria catalyze the fixation of CO_2 to acetyl-CoA. The finding that malonate and bicarbonate are required in a system which apparently cannot metabolize them is difficult to explain at present. Further work is being done to elucidate these effects.

The finding that fat-body homogenates converted acetate into fatty acids and not into cholesterol was expected, since it is known that intact insects cannot synthesize sterols from small molecules (24) and require their addition in the diet. In analogy to the cell-free system of Zebe and McShan (19), the locust fat-body homogenate synthesized all *n* long-chain fatty acids with an even number of carbon atoms and also some unsaturated acids. Palmitic acid contained about 60% of the total label. In contrast to the above-mentioned system, in the locust fat-body preparations all the newly synthesized fatty acids were esterified with glycerol as glycerides and phospholipids. An analysis of the fatty acid components of *Locusta migratoria*, published by Hilditch (25), showed that oleic, linoleic, and linolenic acid amounted to 65% of the total fatty acids. This distribution differs markedly from the distribution of labeling found in the fatty acids which were synthesized *in vitro* by fat-body extracts. It is of interest to test whether under suitable conditions the extracts can convert saturated fatty acids into unsaturated acids, or whether the locust, like higher animals, requires an external supply of linoleic and linolenic acid.

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