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THE METABOLISM OF WAX ESTERS AND TRIACYLGLYCEROLS IN CELL-FREE
PREPARATIONS OF THE MARINE COPEPOD, CALANUS HELGOLANDICUS

by

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SUMMARY

In order to understand the mechanisms giving rise to the uncommonly large amounts of wax esters in marine animals from colder waters we have studied lipid metabolism in the copepod, Calanus helgolandicus. A cell-free preparation of this animal catalysed the incorporation of (1-¹⁴C) palmitic acid into wax esters, triacylglycerols and phospholipids by ATP-dependent processes. The relative rates of incorporation were similar to the relative masses of the lipids in Calanus. The rate of formation of wax esters was very dependent on the concentration of added free fatty alcohol but was only stimulated by adding free fatty acid. The cell-free preparations contain an NADH-palmitaldehyde oxidoreductase and also oxidise fatty alcohol to fatty acid. Freshly caught Calanus contain an active triacylglycerol lipase but have negligible wax esterase. Wax esterase appears after starving Calanus for six days but triacylglycerol lipase activity remains unaltered.

INTRODUCTION

The presence of high concentrations of wax esters in the lipids of a variety of marine animals, including certain of the zooplankton copepods such as Calanus, has been well documented¹. As yet no satisfactory explanation is available why these animals accumulate wax esters in preference to triacylglycerols. In vivo turnover studies with Calanus helgolandicus have shown that both wax esters and triacylglycerols can be used as energy reserves². In deep sea copepods triacylglycerols can be preferentially used during starvation³.

Wax esters biosynthesis has been studied in the plant Brassica⁴, in the fresh water fish, Trichogaster cosby^{5,6}, in the livers of the spur dogfish, Squalus,^{7,8} in deep sea myctophid fish⁹, and in a preputial gland tumour from the mouse^{10,11}. In these systems fatty acid appears to be incorporated into wax ester as its acyl coenzyme A derivative^{4,8,11}. In vivo interconversions of fatty acid and fatty alcohol have been demonstrated in Trichogaster cosby^{5,6},

whereas the reduction of fatty acyl coenzyme A to fatty alcohol in the presence of reduced pyridine nucleotides has been demonstrated in cell-free preparations of Euglena and Brassica^{12,13}, preputial gland tumour¹¹, Clostridium butyricum¹⁴ and Mycobacterium tuberculosis¹⁵. Feeding experiments showed that wax esters can be deposited by Calanus in the absence of significant amounts of dietary fatty alcohols¹⁶.

The present work is concerned with the metabolism of wax esters and triacylglycerols in Calanus in an attempt to understand the mechanisms giving rise to the uncommonly large levels of wax esters in copepods.

MATERIALS AND METHODS

Calanus helgolandicus

Copepods were caught three miles off La Jolla, Calif. during daylight in mid-September. In most cases packed copepods, consisting of more than 90 per cent adult C. helgolandicus, were homogenised immediately. In one experiment live C. helgolandicus were picked from the sample, transferred to filtered sea water

(Millipore filter of 0.45μ) at 18° and starved for six days.

Isotopically labelled compounds

($9,10-^3\text{H}_2$)Palmitic acid (50mCi/m-mole), ($1-^{14}\text{C}$)-palmitic acid (10 mCi/m-mole) and ($1-^{14}\text{C}$)oleic acid (50mCi/m-mole) were purchased from Dhom Products Ltd., No. Hollywood, Calif. The latter compound was reduced to ($1-^{14}\text{C}$)oleyl alcohol with lithium aluminium hydride. Thin layer chromatographic examination of the product showed that more than 99% was the labelled alcohol. (^{14}C)Triolein (glyceryl trioleate- $1-^{14}\text{C}$, 88 $\mu\text{Ci/m-mole}$) was purchased from Tracerlab, Waltham, Mass.

A polyunsaturated wax ester labelled with tritium was synthesised from ($9,10-^3\text{H}_2$)oleic acid (750mCi/m-mole) and crude linoleyl alcohol (73% 18:2, 10% 18:1 and 8.0% 18:3) prepared by lithium aluminium hydride reduction of crude linoleic acid. The final, chromatographically pure material had a specific activity of 460 $\mu\text{Ci/m-mole}$.

($9,10-^3\text{H}_3$)Palmitaldehyde (7.2mCi/m-mole) was

kindly provided by Dr. E. G. Trams of the National Institutes of Health, Bethesda. This material was purified chromatographically to more than 99% purity.

Chemicals

The sodium salts of DL- α -glycerophosphate and ATP were purchased from Sigma Chem. Co., St. Louis Mo. NADPH and Coenzyme A were purchased from P.-L. Biochemicals, Milwaukee, Wis.

Preparation and incubation of cell fractions

(i) Lipid biosynthesis

A homogenate of Calanus (1:1 w/v) in 0.25M sucrose containing 0.1M tris-HCl, pH 7.4, was centrifuged at 1,000g for 10 min. and the supernatant withdrawn for assay. Conditions of individual assays are detailed in Table 1-3. Reactions were stopped by extracting total lipids by the method of BLIGH AND DYER¹⁷.

(ii) Reduction of (9,10-³H₂)palmitaldehyde

A 50:1 (V/W) homogenate of Calanus in 100mM KCl

was centrifuged at 1,000g for 1 min. The supernatant (0.1ml., 0.024mg of protein) was assayed at 25° in a total volume of 0.40ml. with 10μl. of a solution of (9,10-³H₂)palmitaldehyde (2.3μCi, 32nmoles) in 0.2% sodium taurocholate and 0.29ml. of buffer. The latter was either 0.3M potassium phosphate of pH 6.0 or 7.0, or 0.3 M tris-HCl of pH 8.0 or 9.0. NADH were present was added to a final concentration of 2.0 mM. The entire incubation was carried out under nitrogen. After two hours the reaction was stopped by adding 0.1 ml. of 2N HCl and the lipids extracted into hexane.

(iii) Lipase assays

Homogenates of Calanus (10:1 V/W) were prepared in either 0.2 M potassium citrate of pH 6.0, 0.2 M potassium phosphate of pH 7.0, or 0.2 M tris-HCl of pH 8.0. The homogenates were centrifuged at 27,000 g for 10 min. to yield a pellet, a clear middle layer and a floating layer of oil. 0.2 ml. of the middle layer (1.9 mg. of protein) was taken for the following assays. Wax ester lipase assays contained the enzyme together with 0.2 ml. of the appropriate

buffer and 0.2 ml. of a suspension of linoleyl (^3H)oleate (0.2 μCi = 0.23 mg.) in 0.9% NaCl containing 0.08% sodium taurocholate. The contents of each tube were mixed on a vortex mixer for 60 sec. and incubated at 30°C for two hours. For triacylglycerol lipase assays the substrate was (^{14}C)triolein (0.1 μCi = 1.0 mg.). The reaction was terminated by extracting total lipid by the method of DOLE¹⁸. The final hexane phase was extracted with 1.0 ml of 0.1 M KOH, and 0.1 ml. of the resulting aqueous layer assayed for radioactivity.

Lipid separations

Total lipids were separated on thin layers of silicic acid into wax esters and triacylglycerols using petroleum ether : diethyl ether : acetic acid (90:10:1 by volume). Fatty acid, aldehyde and alcohol were separated in petroleum ether : diethyl ether : acetic acid (40:60:4 by volume). In one experiment wax esters were saponified using potassium t-butoxide¹⁶, and the resulting acids and alcohols separated using petroleum ether : diethyl ether : acetic acid (70:30:1 by volume). Phospholipids were separated from total

lipids using the two-dimensional system described by PARSONS AND PATTON¹⁹. Lipids were detected on thin layers by brief exposure of the plates to iodine vapour.

Assay of radioactivity

Lipids were scraped directly from the plates into 10 ml. of Aquasol (New England Nuclear Corp., Boston, Mass.) and assayed in a scintillation counter. In the lipase experiment 0.1 ml. of 0.1 M KOH solution was counted in 10 ml. of a solution of 5 g. PPO and 100 mg. of dimethyl POPOP in 700 ml. of toluene, 300 ml. of methanol and 100 ml. of hyamine hydroxide. All radioassays were carried out using a Beckman Scintillation Counter (model DPM 100) operated at a gain of 290. Under these conditions efficiencies for counting were: channel 1 - tritium 39.4%, carbon-14 22.9%; channel 2 - tritium 0%, carbon-14 60.1%. All samples were counted to an accuracy of $\pm 2\%$ S.E.

RESULTS

Initially the 1,000g supernatant of a homogenate of whole, freshly caught Calanus was incubated with

ATP, magnesium ions and coenzyme A in a buffered medium. (Table 1) Under these conditions (1-¹⁴C) palmitic acid was incorporated into both neutral and polar lipids, the latter being more heavily labelled. Of the neutral lipids wax esters were more heavily labelled than triacylglycerols. The lipid classes in Table 1 were separated by thin layer chromatography. Radioautographic examination of the plates showed that radioactivity coincided with mass and that lipid classes were well separated from one another and from the precursor fatty acid.

TABLE 1

Table 2 shows that incorporation of radioactivity into both wax esters and triacylglycerols is markedly stimulated by adding ATP together with coenzyme A. Radioautographic examination of the plates from these experiments showed that incorporation into polar lipids was also stimulated by adding ATP and coenzyme A. Adding DL- α -glycerophosphate or 2-monolein did not markedly stimulate incorporation of radioactivity into triacylglycerols, whereas adding oleyl alcohol strikingly stimulated incorporation into wax esters.

TABLE 2

In the latter experiment oleyl alcohol was added dispersed in an excess of Triton X-100. This detergent did not materially alter the extent of incorporation on its own. Boiling the cell fraction abolished its activity.

Table 3 confirms some of the data in Table 2 by showing that incorporation of (^3H)palmitic acid into wax ester is increased by adding ATP and coenzyme A, as well as being markedly stimulated by adding oleyl alcohol. Table 3 shows additionally that incorporation of (^{14}C)oleyl alcohol into wax ester is much less dependent on adding ATP, presumably reflecting the presence of a small pool of fatty acyl coenzyme A in the system. Furthermore the incorporation of oleyl alcohol into wax ester is only stimulated some 45% by adding palmitic acid. These results demonstrate that the formation of wax ester in the 1,000g supernatant is seriously limited by the concentration of free fatty alcohol but is only slightly limited by the concentration of free fatty acid.

TABLE 3

Saponification of the wax ester from the system in Table 3 incubated with both (^{14}C)oleyl alcohol and (^3H)palmitic acid and separation of the acid and alcohol moieties on thin layers, showed that more than 95% of the tritium and carbon-14 were recovered in fatty acid and fatty alcohol respectively. Consequently under the conditions used both fatty acid and fatty alcohol are directly incorporated into wax ester without undergoing significant interconversion. That conversion of fatty alcohol to fatty acid does occur in the system, however, is shown by the presence of carbon-14 in triacylglycerols after incubating with (^{14}C)oleyl alcohol. Examination of the positional distribution of radioactivity in triacylglycerols formed in Table 3 by the method of LUDDY *et al.*²⁰ showed that 17% of the total radioactivity was in position 2 and the remaining 83% was in positions 1 and 3.

Table 4 shows that the 1,000g supernatant of the homogenate of Calanus will convert (^3H)palmitaldehyde to palmityl alcohol. These assays were carried out under nitrogen to minimise atmospheric oxidation of

TABLE 4

the aldehyde to free acid and only very slight enzymic and non-enzyme oxidation of aldehyde to acid actually occurred. In contrast, formation of palmityl alcohol was catalysed by a heat labile component in the 1,000g supernatant and such formation was stimulated more than two fold by adding NADH at the optimal pH of 7.0.

Table 5 shows that fractions of freshly caught Calanus contain little or no wax esterase as judged by their ability to hydrolyse linoleyl (³H)oleate. An active triacylglycerol lipase is present as judged by the hydrolysis of ¹⁴C-labelled triolein. The latter activity is unchanged after starving the live animals for six days, whereas a significant wax esterase activity appears after this time. Nonetheless the triacylglycerol lipase is still five times more active than the wax esterase. Both enzymes are more active at pH 7.0 than at pH 6.0. The data show that triacylglycerols are preferentially hydrolysed over wax esters in the fractions of Calanus used in the present experiments.

TABLE 5

DISCUSSION

Calanus may contain up to one half of its dry weight as lipid; wax esters, triacylglycerols and phospholipids constitute some 40%, 5-10% and 45% of the total lipids respectively^{2,16,21}. Both oleyl alcohol and palmitic acid are prominent constituents of the wax esters and C34 esters form a considerable proportion of the total waxes¹⁶.

The present work was carried out with homogenates of whole animals, i.e. the cell-free preparations are derived from all cell types including those from the digestive tract. Despite this the preparations incorporated radioactive fatty acid into all the major lipid classes of Calanus when supplemented with cofactors including ATP and coenzyme A. Indeed the relative levels of radioactivity incorporated into wax esters, triacylglycerols and phospholipids are similar to the relative masses of these lipids in Calanus (Table 1, 2, 3). Of the radioactivity added as palmitic acid in Table 1 only some 0.3% is incorporated into wax ester. However, when the system is supplemented with oleyl

alcohol some 2.7% of the added radioactivity is incorporated (Table 2). It should be noted that the lipids of Calanus contain about 1% of their weight as free fatty acids while negligible levels of free fatty alcohols are present (LEE, R.F. unpublished). We have shown, moreover, that the incorporation of free fatty alcohol into waxes is not appreciably stimulated by adding free fatty acids (Table 3). These considerations make it highly likely that the free fatty acid concentration in the fractions of Calanus used is essentially saturating with respect to wax ester synthesis. Consequently the figure of 2.7% of added palmitic acid incorporated is by no means inconsistent with a relatively active process of wax ester biosynthesis occurring.

All the data in the present work are consistent with the biosynthesis of wax ester from free fatty acid and free fatty alcohol by a reaction dependent on ATP and coenzyme A (Tables 1, 2, 3). Such a reaction is highly likely to proceed by way of fatty acyl coenzyme A. The biosynthesis of wax ester via fatty acyl coenzyme A has been well substantiated in

Brassica⁴, Euglena¹², Squalus liver⁸, a mammalian tumour¹⁰ and Mycobacterium tuberculosis¹⁵. Preparations of marine copepods will incorporate [¹⁴C]palmityl CoA directly into wax esters (SARGENT, J.R. unpublished).

The present work shows that the rate of formation of wax ester in the 1,000g supernatant is markedly limited by the concentration of free fatty alcohol present (Tables 2, 3) and is scarcely limited by the concentration of free fatty acid present (Table 3). Thus it is the concentration of acyl acceptor that determines the rate of wax ester biosynthesis. This situation may be contrasted with that obtained for triacylglycerols where addition of DL- α -glycerophosphate or 2-monoolein does not appreciably stimulate incorporation of radioactivity into the lipid (Table 2). It would appear that incorporation of radioactivity is limited by the activity of the esterifying enzymes in the latter case. Since the concentration of free fatty alcohol limits the formation of wax ester it can be simply stated that the rate of formation of fatty alcohol is the rate limiting reaction in wax ester

biosynthesis from fatty acids. The formation of fatty alcohol from fatty acid has been documented in a number of systems including Brassica¹², Euglena¹³, preputial gland tumour¹¹, Clostridium¹⁴ and Mycobacterium¹⁵.

In several of these systems^{12, 13, 14, 15} fatty aldehyde has been shown to be an intermediate in the reduction of fatty acyl coenzyme A in the presence of reduced pyridine nucleotides. The present work shows that an NADH-palmitaldehyde oxidoreductase exist in Calanus (Table 4) and it is probable that this activity is associated with the conversion of fatty acid to fatty alcohol. The present results also present indirect evidence for the oxidation of fatty alcohol to fatty acid in the sense that (¹⁴C)oleyl alcohol serves as a precursor for triacylglycerols (Table 3). The latter result is noteworthy in that (¹⁴C)oleyl alcohol does not serve as a precursor of the fatty acyl moieties of wax esters (p. 8). Consequently at least two different pools of fatty acids involved in ester bond formation are present in the 1,000g supernatant.

Such pools may well be a result of sub-cellular compartmentation of the reactions involved.

We have shown that the fractions of Calanus used here synthesise wax esters faster than triacylglycerols. Table 5 shows that freshly caught, and presumably well fed Calanus hydrolyse triacylglycerols readily but not wax esters. Wax esterase activity appears, however, after six days starvation although triacylglycerol lipase is still the more active. This result can be interpreted as showing that wax esters constitute an energy reserve that is relatively conserved in Calanus. The products of wax ester hydrolysis are free fatty acid and free fatty alcohol and the latter is known to be a potent inhibitor of triacylglycerol lipase²². Indeed fatty alcohols may be anticipated to be efficient inhibitors of the hydrophobic active centres of enzymes involved in lipid metabolism generally. As stated earlier, however, the present work shows that Calanus has the ability to oxidize fatty alcohols to fatty acids. The presence of a negligible pool of free fatty alcohols in Calanus lipid implies that the rate

of oxidation of fatty alcohol of fatty acid is faster than the rate of production of fatty alcohol from wax ester. This situation is entirely in keeping with the earlier conclusion that the rate of formation of wax ester exceeds the rate of formation of fatty alcohol from fatty acid.

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TABLE 1

INCORPORATION OF (^{-14}C)PALMITIC ACID INTO LIPIDS IN A CELL-FREE PREPARATION OF CALANUS

0.1ml. of the 1,000g supernatant (1.2mg. of protein) was incubated for 4 hours at 25° in a total volume of 0.5ml. with 10mM ATP, 10mM MgCl_2 , 0.5mM coenzyme A, 0.01mM oleyl alcohol, 0.25M sucrose, 0.1M tris-HCl pH 7.4 and 0.3 μCi of (1^{-14}C)palmitic acid (10mCi per m-mole). The latter lipid and oleyl alcohol were dispersed in 10 μl . of 2 per cent Triton X-100. Lipids were isolated and assayed for radioactivity as described in the Methods. Values shown are the means of duplicate determinations.

LIPID CLASS	d.p.m./assay
Wax Ester	3430
Triacylglycerol	1570
Phosphatidylcholine	6000
Phosphatidylethanolamine	1330
Sphingomyelin	700
Lysophosphatidylethanolamine	167

TABLE 2

EFFECT OF COFACTORS ON THE INCORPORATION OF (1-¹⁴C)PALMITIC ACID INTO
WAX ESTERS AND TRIACYLGLYCEROLS IN A CELL-FREE SYSTEM FROM CALANUS

Conditions of assay are essentially as described in Table 1. Oleyl alcohol and 2-monoolein were added after dispersion in 0.1ml. of a pH 7.4 solution (0.1M tris-HCl) of 0.25M sucrose containing 2 per cent Triton X-100. Values shown are means of duplicate determinations.

COFACTOR	WAX ESTERS d.p.m./assay	TRIACYLGLYCEROLS d.p.m./assay
ATP, CoASH	3,250	1,480
ATP omitted, CoASH omitted	740	260
ATP, CoASH, 100mM DL- α -glycerophosphate	3,620	1,750
ATP, CoASH, 0.05mM 2-monoolein	3,800	1,500
ATP, CoASH, 0.05mM oleyl alcohol	8,500	2,880
ATP, CoASH, 1.0mM oleyl alcohol	29,670	1,538
ATP, CoASH, 1.0mM oleyl alcohol (boiled enzyme)	130	100

TABLE 3

INCORPORATION OF (^{14}C)OLEYL ALCOHOL AND (^3H)PALMITIC ACID INTO WAX ESTER
AND TRIACYLGLYCEROL IN A CELL-FREE SYSTEM FROM CALANUS

Conditions of assay are essentially as described in Table 1. Either $10\mu\text{Ci}$ of (9,10- $^3\text{H}_2$)palmitic acid (50mCi per m-mole) or $2\mu\text{Ci}$ of (1- ^{14}C)-oleyl alcohol (50mCi per m-mole), or both, were added along with unlabelled oleyl alcohol and unlabelled palmitic acid as shown. The lipid precursors were added in $60\mu\text{l}$. of 2 per cent Triton X-100 containing 0.1M tris-HCl pH 7.4 and 0.25M sucrose after dispersion by sonification. Values shown are means of duplicate determinations.

LIPID PRECURSORS	ATP coenzyme A	WAX ESTERS		TRIACYLGLYCEROLS	
		d.p.m. $\times 10^{-3}$		d.p.m. $\times 10^{-3}$	
		per assay		per assay	
		^3H	^{14}C	^3H	^{14}C
(^{14}C)oleyl alcohol (0.5mM)	present	---	87	--	--
(^{14}C)oleyl alcohol (0.5 mM) + palmitate (0.5mM)	present	---	126	--	13
(^3H)palmitate	present	127	---	31	--
(^3H)palmitate + oleyl alcohol (0.5mM)	present	592	---	40	--
(^{14}C)oleyl alcohol (0.5mM) + (^3H)palmitate	absent	188	64	26	12
"	present	515	97	33	8

TABLE 4

FORMATION OF (³H)PALMITYL ALCOHOL FROM (³H)PALMITALDEHYDE IN A CELL-FREE SYSTEM FROM CALANUS

The 1,000g supernatant (0.024mg. of protein) of a homogenate of Calanus was incubated for 2 hours at 25° with 2.3μCi of (9,10-³)palmitaldehyde (7.2mCi/m-mole) and radioactivity determined in palmityl alcohol and palmitic acid. Full details of the assay are given in the Methods. Values shown are the means of duplicate determinations.

pH	CELL FRACTION	NADH	ALCOHOL	ACID
			d.p.m. x 10 ⁻³ per assay	d.p.m. x 10 ⁻³ per assay
6.0	-	-	40	32
	+	-	470	92
	+	+	700	64
7.0	-	-	41	44
	^a +	-	54	38
	+	-	280	64
	+	+	720	61
8.0	-	-	39	38
	+	-	350	71
	+	+	490	63
9.0	-	-	36	39
	+	-	80	22
	+	+	89	23

^aEnzyme fraction heated at 100° for 5min.

TABLE 5

WAX ESTER AND TRIACYLGLYCEROL LIPASE ACTIVITIES IN CELL-FREE PREPARATIONS OF FRESHLY CAUGHT CALANUS, AND CALANUS STARVED FOR SIX DAYS

Cell-free fractions of Calanus were incubated with linoleyl (^3H)oleate or (^{14}C)triolein at 30° for 2 hours and radioactivity determined in free fatty acids recovered from the system. Full details are given in the Methods. Values shown are the means of duplicate determinations.

ENZYME SOURCE	pH	nmoles of substrate hydrolysed/mg. protein	
		oleyl oleate	triolein
fresh <u>Calanus</u>	7	0.1 ^a	1.0 ^a
	7	0.2	43.0
	6	0.1	39.0
starved <u>Calanus</u>	7	2.7 ^a	1.1 ^a
	7	8.7	44.0
	6	5.5	41.0

^aData refer to an enzyme fraction heated at 100° for 5 min. Other values in the Table have been corrected for these controls.