The metabolism of wax esters and other lipids by the marine copepod, *Calanus helgolandicus*

RICHARD F. LEE, JUDD C. NEVENZEL, G.-A. PAFFENHÖFER, and A. A. BENSON

Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92037

ABSTRACT The polyunsaturated wax esters which occur in an oil droplet in *Calanus helgolandicus* apparently serve as a short term reserve metabolic fuel. The lipids of the copepods were labeled by feeding them ^{14}C-labeled diatoms, *Skeletonema costatum*, after which starvation and turnover experiments were carried out. During starvation both wax esters and triglycerides are largely depleted within 72 hr, whereas cholesterol and phospholipid decreased at much slower rates.

SUPPLEMENTARY KEY WORDS phospholipids · cholesterol · crustaceans · triglycerides · starvation

PREVIOUS WORK by the authors has shown the presence of wax esters in *Calanus helgolandicus* (1). The composition of the wax esters was found to vary with the nutritional state of the animals indicating that wax esters were undergoing rapid metabolic changes. In order to study these metabolic changes, we fed ^{14}C-labeled diatoms, *Skeletonema costatum*, to adult female *Calanus* and observed the changes which occurred in the lipids during subsequent starvation or feeding with nonlabeled diatoms.

Earlier workers also used radioisotopes to study the ingestion and metabolism of food by small crustaceans of the zooplankton. Marshall and Orr (2) fed ^{14}C-labeled diatoms to *Calanus finmarchicus* and found high utilization rates for ingested food. Using ^{32}P-labeled diatoms (3), they found that phospholipid, which accounted for 40% of the ^{32}P in the diatoms, was utilized by *Calanus* during starvation. high uptake rates were also reported by Lasker (4) for a ^{14}C-labeled green alga, *Dunaliella primolecta*, fed to *Euphausia pacifica*.

MATERIALS AND METHODS

100 ml of a 6 day culture of the diatom *Skeletonema costatum* was labeled with $^{14}CO_2$ in a 2 liter desiccator by adding lactic acid to a small open vial containing 8 mCi of $BaCO_3$-^{14}C. The culture was illuminated for 2 days with fluorescent lights (approximately 600 ft-c). The labeled diatoms were then placed in a 2 liter beaker containing 24 adult female *Calanus* in filtered seawater. The diatom concentration in the beaker was equivalent to approximately 400 μg of carbon per liter. The *Calanus* were fed the labeled diatoms for 48 hr; samples for analysis (three individuals each) were taken at 24 and 48 hr. After 48 hr, nine *Calanus* (Group A) were removed and fed for 3 days on nonlabeled diatoms, while nine other *Calanus* (Group B) were removed and starved for 3 days. There was no mortality during the experiments.

During the turnover and starvation experiments, the *Calanus* lipid was sampled each 24 hr period by removing three animals from the beaker and by extracting them with chloroform–methanol 2:1 (v/v). The lipid extracts were dried under nitrogen and then dissolved in 0.5 ml of hexane. 10 μl samples were counted and applied to thin-layer plates (Silica Gel G; Merck & Co., Inc., Rahway, N.J.). Thin-layer plates were developed in petroleum ether–ethyl ether 70:30 (v/v) for the analysis of the neutral lipid, while for phospholipid determination chloroform–methanol–water 65:25:4 (v/v/v) was used. Radioautographs of the thin-layer plates were carried out using Kodak Blue Sensitive Medical X-ray Film. The film was developed after 24 hr exposure. The position of the different classes of lipids such as wax, triglyceride, sterol, and phospholipid was then determined, and each was scraped off and added to a suspension of 5% Cab-O-Sil and 0.5% PPO (2,5-diphenyloxazole; Packard Instrument Co., Downers Grove, Ill.) in toluene for counting in a liquid scintillation counter.

Since three *Calanus* contain only 120 μg of lipid, we fractionated by silicic acid column chromatography the lipids of 200 unlabeled laboratory *Calanus* into hydrocarbons, wax esters, triglycerides, sterols, and phospho-

lipids (5). For the laboratory *Calanus*, fed 400 μg of carbon per liter, the following by weight composition was observed: hydrocarbons, 1%; wax esters, 37%; triglycerides, 12%; sterols, 16%; and phospholipids, 33%. The weight of each fraction was used for the calculation of specific activities. Because the weight of the lipid from starved animals was so small, the specific activity of each lipid class could not be calculated for this group of animals. Trifluoroacetates of the sterol fraction were prepared by the procedure of Wood and Snyder (6) and then analyzed by gas–liquid chromatography. We used a 1.8 m × 3.2 mm (O.D.) column of 3% OV-1 on 60–80 mesh Gas-Chrom P in the Loenco Model 70 Hi-Flex apparatus fitted with a flame ionization detector and operated isothermally at 250°C and 2.1 kg/cm² nitrogen carrier gas pressure. Two peaks of different size were observed; the major peak had the same retention time as cholesterol (21.0 min), while the minor peak was suspected to be an isomer of cholesterol because of the close retention time (22.0 min).

The *Calanus* used for the experiments were reared from the eggs at a diatom concentration of 400 μg of carbon per liter. A more detailed description of our method of rearing *Calanus* is found in the paper by Paffenhöfer (7). The wild *Calanus* used in certain starvation experiments were captured in a plankton net towed at a depth of 25 m approximately 5 miles offshore.

RESULTS AND DISCUSSION

The amount of lipid was found to decrease rapidly in starved animals (Table 1). Note that there is a much less rapid decrease in lipid content for copepods collected at sea ("wild type") as compared with laboratory-grown animals. A possible explanation may be that the wild animals are metabolically adjusted to rapid changes in the availability of food, whereas the laboratory-raised animals were not adapted to oscillations of food supply. A second possibility may be that the general health of the laboratory-raised animals is impaired, and so they cannot withstand the stress of starvation as well as wild animals. However, the time required to reach the adult stage and the ultimate size of the animals were the same for both laboratory-raised and wild *Calanus*. In addition, Mullin and Brooks (8) have shown that the respiratory rate of *Calanus* fed on the diatom *Thalassiosira* was the same for wild copepods as for laboratory-grown animals. Thus we conclude that the first hypothesis should be investigated further.

The *Skeletonema costatum* fed to the copepods contained a total of 4.32×10^9 cpm of ^{14}C. After a 48 hr feeding (400 μg of carbon per liter), the *Calanus* has 2.12×10^6 cpm per animal. Assuming uniform labeling of all carbon compounds, this gives a value of 21.9% for the lipid

TABLE 1 THE EFFECT OF STARVATION ON LIPIDS OF COPEPODS

Days of Starvation	Group I	Group II
	% lipid	
0	18.1	15.4
1	9.0	
2	6.0	
3	4.2	
7	1.5	7.7

Group I copepods were fed on *Skeletonema* (400 μg of carbon per liter) until the start of the starvation experiment. Group II copepods were collected approximately 5 miles offshore and then starved.

content of these copepods; a gravimetric determination was 18.1%. A similar calculation for the lipid content of the *Skeletonema* from the radioactivity data indicated 8.3% lipid, compared with 8.1% as determined gravimetrically. From these same radioactivity data, noting that a total of 800 μg of carbon was available to the copepods, and assuming that 30% of the carbon from the diatoms was retained while 70% was excreted or oxidized to CO_2 (8), we calculate that 10.8 μg of carbon per copepod per day was assimilated. This figure may be compared with Mullin's value of 15 μg carbon per copepod per day for adult female *Calanus* raised at 15°C on *Thalassiosira*.[1]

Petipa (9) determined the amount of lipid in *Calanus helgolandicus* by measuring the volume of the fat droplet. There appeared to be a diurnal rhythm of lipid production, with feeding and lipid accumulation at night and utilization of the lipid droplet during the day. She postulated that the lipid droplet formed during the night feeding served as energy for the downward migration during the day. By feeding *Nitzschia* species she found that lipid droplet formation occurred at approximately 135 μg of carbon per liter (10). Our own work with *Skeletonema costatum* indicated that lipid droplet formation does not occur below 50 μg of carbon per liver. We have previously noted that the lipid droplet was mostly wax ester (1), and since the wax ester shows a high turnover rate (see Table 2), Petipa's idea of the lipid droplet fluctuating diurnally has merit. Marshall and Orr showed that when *Calanus* was kept in small containers it fed both day and night (11); consequently no diurnal variation in lipid would be expected in our experiments. Linford (12), from starvation experiments, and Marshall and Orr (11), from seasonal studies, have concluded that lipid cannot be used as an energy resource. We disagree with this conclusion because of the observed variation of lipid with diatom concentration (1) and the decrease in lipid occur-

[1] Personal communication from M. Mullin (Scripps Institution of Oceanography, La Jolla, Calif.).

TABLE 2 CHANGES IN ^{14}C-LABELED LIPIDS OF *Calanus**

Time†	Group A (Unlabeled Diet) Activity	Group A (Unlabeled Diet) Specific Activity	Group B (Starved) Activity
hr	*cpm* × 10^{-2}	*cpm/mg* × 10^{-3}	*cpm* × 10^{-2}
I. Wax ester			
24	95	190	95
48	160	330	160
72	140	290	18
96	89	180	7
120	42	80	2
II. Triglyceride			
24	140	950	140
48	220	1400	220
72	210	1400	25
96	130	870	12
120	54	350	3
III. Phospholipid			
24	140	53	140
48	410	960	410
72	380	880	160
96	280	700	160
120	210	490	84
IV. Cholesterol			
24	8	37	8
48	32	150	32
72	22	110	18
96	19	92	17
120	23	110	17

The copepods were fed on ^{14}C-labeled *Skeletonema costatum* for 48 hr and were then divided into two groups. Group A was fed unlabeled *Skeletonema* for 3 days, while Group B was starved for 3 days. The total time of experiment was 120 hr.

* 24 copepods were used for the experiment, and three animals were sacrificed for each time interval.

† Measured from start of ^{14}C-*Skeletonema* feeding.

TABLE 3 CHANGES IN RATIOS OF ^{14}C ACTIVITY IN WAX/TRIGLYCERIDE AND WAX/PHOSPHOLIPID DURING STARVATION AND TURNOVER EXPERIMENTS IN *Calanus**

Time	Wax/Triglyceride	Wax/Phospholipid
hr		
I. Turnover experiment (Group A copepods)		
48	0.74	0.39
72	0.68	0.38
96	0.66	0.32
120	0.76	0.20
II. Starvation experiment (Group B copepods)		
48	0.65	0.39
72	0.70	0.11
96	0.58	0.04
120	0.61	0.02

* Data are derived from total activities in Table 2.

ring during our starvation experiments (see Table 1). Work with several euphausids has indicated dramatic lipid changes which are correlated with season (13–15). Although *Calanus* does not form a lipid droplet with 50 μg of carbon per liter, they appear to be healthy, if somewhat smaller in size (7).

Assuming that the drop in total ^{14}C activity of the various lipid fractions is a direct measure of the decreasing amounts of these materials in the starved animals, we deduce that the wax and triglyceride decreases are parallel to the drop in lipid content (Tables 2 and 3). The phospholipids and cholesterol do not drop as sharply (Tables 2 and 3). Thus during starvation wax esters and triglycerides are utilized to a greater extent than either phospholipids or cholesterol. This is probably because phospholipids and cholesterol have a structural function in membrane (16, 17), whereas triglycerides serve for energy storage (17, 18). Since the wax/triglyceride ratio remains constant during starvation (see Table 3), it appears that wax esters can be used for energy as effectively as triglycerides. The saturated or monounsaturated waxes deposited on leaf surfaces (19, 20) and secreted by mammalian sebaceous glands (21) have been assumed to have slow turnover rates. The polyunsaturated wax esters of *Calanus* (1) can be effectively and quickly mobilized to serve the energy requirements of *Calanus*.

This research was supported by Grant GM-12310 from the National Institute of General Medical Sciences, AEC Contract AT (11-1)GEN-10, PA-20, and AEC Contract AT (04-1) GEN-12.

Manuscript received 8 December 1969; accepted 9 February 1970.

REFERENCES

1. Lee, R. F., J. C. Nevenzel, and G.-A. Paffenhöfer. 1970. *Science* (*Washington*). **167:** 1510.
2. Marshall, S. M., and A. P. Orr. 1955. *Deep-Sea Res. Oceanogr. Abstr.* **3** (Suppl.): 110.
3. Marshall, S. M., and A. P. Orr. 1961. *J. Mar. Biol. Ass. U.K.* **41:** 463.
4. Lasker, R. 1960. *Science* (*Washington*). **131:** 1908.
5. Nevenzel, J. C., W. Rodegker, and J. F. Mead. 1965. *Biochemistry*. **4:** 1589.
6. Wood, R., and F. Snyder. 1966. *Lipids*. **1:** 63.
7. Paffenhöfer, G.-A. 1970. *Helgolaender Wiss. Meeresunters.* In press.
8. Mullin, M. M., and E. Brooks. 1970. International Symposium on Marine Food Chains, University of Aarhus, Denmark, July 1968. In press.
9. Petipa, T. S. 1964. *Dokl. Akad. Nauk. SSR.* **156:** 1440.
10. Petipa, T. S. 1964. *Dokl. Akad. Nauk. SSR.* **155:** 470.
11. Marshall, S. M., and A. P. Orr. 1965. The Biology of a Marine Copepod, *Calanus finmarchicus* (Gunnerus). Oliver & Boyd Ltd., London, England. 18–20.
12. Linford, R. 1965. *J. Cons. Cons. Perma. Int. Explor. Mer.* **30:** 16.
13. Raymont, J. E. G., R. T. Srinivasagam, and J. K. B. Raymont. 1969. *Deep-Sea Res. Oceanogr. Abstr.* **16:** 141.
14. Littlepage, J. L. 1964. *Actual. Scient. Ind.* **1312:** 463.
15. Vinogradova, Z. A. 1960. *Dokl. Akad. Nauk. SSR.* **133:** 680.
16. Benson, A. A. 1966. *J. Amer. Oil Chem. Soc.* **43:** 265.

17. Bartley, W., L. M. Birt, and P. Banks. 1968. The Biochemistry of Tissues. John Wiley & Sons, Inc., New York. 127.
18. Gunstone, F. D. 1967. An Introduction to the Chemistry and Biochemistry of Fatty Acids and Their Glycerides. Chapman & Hall Ltd., London, England. 2.
19. Doby, G. 1965. Plant Biochemistry. Interscience Publishers Ltd., London, England. 325.
20. Kolattukudy, P. E. 1968. *Science* (*Washington*). **159:** 498.
21. Nicolaides, N. 1965. *J. Amer. Oil Chem. Soc.* **42:** 708.