A PRELIMINARY RADIOISOTOPE STUDY OF THE FATE OF INGESTED PALMITIC ACID IN THE ANTARCTIC KRILL, *EUPHAUSIA SUPERBA* DANA.

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ABSTRACT. The fate of dietary palmitic acid was followed in male and female Antarctic krill, *Euphausia superba* Dana, by allowing them to ingest a microencapsulated diet containing [^{14}C]palmitate in cod-liver oil. The ratio of label incorporated into the hepatopancreas to that in the muscle reached a steady state after 4 hours. In male krill 65% of label was located in the hepatopancreas and 35% in the muscle. In female krill the proportions of total activity in the hepatopancreas, ovary and muscle were 59%, 11% and 30% respectively. The lipid class distribution varied with both sex and tissue, but in most cases 90% of the label was located in the triacylglycerol and phospholipid fractions. Very little activity was recovered from the free fatty acid fraction, indicating that ingested palmitate was rapidly esterified. No activity could be detected in wax ester, suggesting that the small amounts of wax in adult *E. superba* are not metabolically active, and may be merely the result of ingesting the occasional copepod. Data from ingestion studies with ^{14}C-labelled algae were variable and difficult to interpret.

INTRODUCTION

The use of radioactivity labelled compounds has been a vital tool in the elucidation of biochemical pathways in a wide variety of organisms. This technique has, however, proved somewhat intractable in studies of pelagic marine invertebrates, mainly because of the difficulty of introducing the labelled compound into the organism in a biologically meaningful way. Previous studies have used incubation *in vitro* of isolated tissues, incubation of whole organisms in seawater containing isotope, allowing the organism to feed on starch particles coated with labelled fatty acid and direct injection.

The ideal way of introducing label would be to use natural food items containing only the compound of interest labelled. Unfortunately algal food grown in the presence of ^{14}C or ^{3}H will have an enormous range of compounds isotopically labelled and will thus be of limited value for tracer experiments. Morris and Sargent (1973) coated [1-^{14}C]palmitic acid on to starch granules (potato powder) and fed these to three species of midwater crustacean. A similar technique was used by Morris and others (1973) on *Neomysis integer*. Substantial quantities of label were incorporated, but there were wide variations in results between individuals, and it is not clear whether this reflected variations in feeding rate or whether an unnatural balance between carbohydrate and lipid in the diet may have influenced the pattern of incorporation observed. Direct injection of label is unsatisfactory because it is injurious.

In this preliminary study Antarctic krill, *Euphausia superba* Dana, were allowed to feed on a microencapsulated diet containing [1-^{14}C]palmitate carried in cod-liver oil. Although not a natural food, this diet contained roughly 45% protein and 15% cod-liver oil and was thus considered to be a suitable food. Microencapsulated foods have been successfully used to rear penaeid shrimp from nauplius to mature adult, and so

may be regarded as a satisfactory diet (D. A. Jones, pers. comm). The results obtained were compared with data from krill fed ^{14}C-labelled algae.

Materials and Methods

The microencapsulated diet was manufactured by emulsifying the dietary components together with [1-^{14}C]palmitate (Amersham, 1.85–2.2 GBq/mmol) in a non-aqueous medium containing a natural surfactant. Active sites on the protein molecules contained within the diet were then crosslinked by interfacial polymerization using an acid chloride. The reaction was terminated and the crosslinked protein microcapsules were freeze-dried. The microcapsules were kindly prepared by Dr D. A. Jones of the Marine Science Laboratories, Menai Bridge, North Wales. They were between 5 and 25 μm in size and contained 8370 dpm per mg (502.21 kBq/mg). The final specific activity of the palmitic acid, when diluted with that in the cod-liver oil, was approximately 35 MBq/mg (130 kBq/mmol). Microcapsules were swollen immediately before feeding experiments by stirring for 12 hours in 1 μm-filtered seawater at 4 °C; 6% of the total activity was recovered in the seawater after swelling. Experiments with microencapsulated diets containing [^{14}C]alanine, [^{14}C]glucose and [^{14}C]starch were unsuccessful; in all these cases more than 95% of the label contained in the dry microcapsules was recovered in the seawater after only 60 seconds of swelling.

^{14}C-labelled algae were prepared by growing a mixed sample of Antarctic phytoplankton in the presence of [^{14}C]bicarbonate. These were fed to experimental krill after one week of culture.

Antarctic krill, *Euphausia superba*, were collected by 1 m Isaacs–Kidd midwater trawl from the Gerlache Strait, Antarctic Peninsula, and transported in aquaria to Palmer Station in December 1984. Krill were maintained in aquaria at 1 ± 0.5 °C, through which was flowing seawater which had been filtered through sand. This population was actively moulting and feeding, and was supplemented at intervals with individuals caught close to the base with dip-nets.

For feeding trials krill were carefully transferred to glass chambers containing 1.2 litres of 1 μm-filtered seawater. Each chamber contained 2 krill, and 4 chambers were used for most experiments. Temperature control was maintained by floating the experimental chambers in the holding tanks. To each experimental chamber was added 30 ml of microcapsules suspended in seawater (containing about 12 mg and 10^5 dpm ^{14}C) or 30 ml algal suspension, followed by 1 ml of 1 mM citric acid to stimulate feeding; the chamber was then covered with foil. After 4 hours the krill were removed, washed briefly in seawater, measured and weighed. The krill were then dissected into three fractions, hepatopancreas, ovary (if present) and the remainder; the latter fraction contained any haemolymph released and consisted largely of cuticle and muscle. This fraction was termed 'muscle'. Lipid was extracted according to Bligh and Dyer (1959) and dissolved in 0.4 ml chloroform; 0.25 ml was counted to estimate total incorporated activity and a subsample of the remainder separated by TLC to determine lipid class distribution of label. The lipid was applied as a band of 1 cm length to a pre-coated plate of silica gel H. The plate was developed in petroleum spirit (40–60°)/diethyl ether/acetic acid 80/20/1 (v/v) and the lipid bands rendered visible with iodine vapour. The bands were outlined and the iodine allowed to evaporate; the marked zones were then scraped into scintillation vials and counted. The lipid classes counted for each sample were hydrocarbon, sterol ester + wax ester, triacylglycerol, diacylglycerol, free fatty acid, cholesterol, monoacylglycerol and phospholipid. Control krill were held in aquaria with no added microencapsulated

food and were fully analysed alongside experimental krill. Two control krill were run for every experimental run of 8 krill fed labelled food.

Total lipid samples were blown free of solvent with a slow stream of oxygen-free dry nitrogen, dissolved in 15 ml Aquasol and counted. Fractions from silica gel plates were counted in a gel of 10 ml Aquasol and 5 ml water. To determine the specific activity of the capsules, known weights (about 10 mg) were digested in 1 ml Protosol and then counted following neutralization with 0.5 ml 1 N-HCl and the addition of 14 ml Aquasol. Preliminary counts (counts/min) were obtained at Palmer Station on an LKB Rackbeta counter. Samples were then returned to Cambridge and re-counted on a Nuclear Chicago Mk III counter. Efficiencies were 78–81 % for both liquid and gel samples, and all data have been corrected to disintegrations per minute (dpm). Appropriate blanks and controls were run with all experiments (Table I).

Table I. Blank, control and recovery details. All blank data expressed as disintegrations per minute (dpm). Control data given as dpm observed after 4 hours incubation, corrected for blank values. SE, standard error; *n*, number of replicates or determinations; M, male; F, female

	Activity (dpm)		
	Mean	*SE*	*n*
Blanks			
Scintillant (Aquasol)	15.6	0.5	17
Digestion (Protosol + Aquasol)	17.5	0.8	15
Silica gel in Aquasol	25.0	1.2	9
Controls (corrected for blanks)			
M Hepatopancreas	6.2	3.3	4
Muscle	6.9	3.4	4
F Hepatopancreas	14.9	9.0	4
Ovary	5.4	4.7	4
Muscle	7.4	3.7	4
Recovery of ^{14}C-labelled lipid from silica gel (%)	97.2	3.6	37

Results

Time course of incorporation

In order to determine a suitable time for experimental incubation, the time course of incorporation of ingested [^{14}C]palmitate into hepatopancreas and muscle was studied. Four aquaria, each containing two male krill (all freshly sampled from the same swarm in Arthur Harbour), were inoculated with equal amounts of labelled microcapsules. Krill were then sampled at intervals up to 4 hours later, and the lipid isolated separately from the hepatopancreas and muscle, and the incorporated label counted.

The overall amount of ^{14}C incorporated into lipid (hepatopancreas and muscle data combined) showed no significant relationship with incubation time (one-way ANOVA, $F_{3,4} = 0.34$, $P > 0.05$). Although there was a tendency for high levels of incorporation of ^{14}C label to be found at longer incubation times, any underlying trend was obscured by enormous individual variation in feeding activity (Fig. 1). This variation was a feature of all the experiments, as has been found in previous studies.

Ingested lipid will pass first to the hepatopancreas, where some will be stored. Some lipid will be used almost immediately for fuel and the remainder may be transported elsewhere in the body either for fuel, turnover or storage. In the time-course study

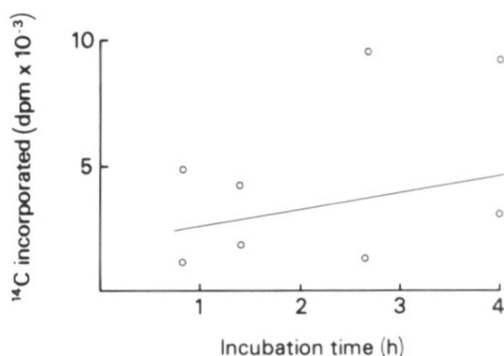

Fig. 1. Time course of incorporation of [^{14}C]palmitate into male *Euphausia superba*. The slope of the regression line is not significantly different from zero ($P > 0.05$).

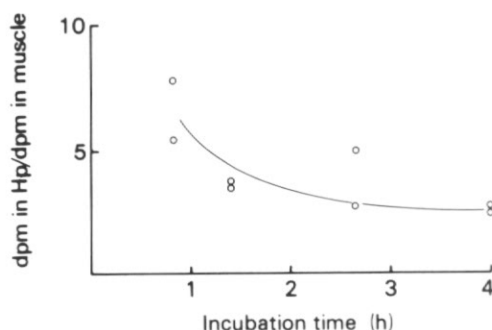

Fig. 2. Variation of tissue distribution of [^{14}C]palmitate in male krill, *Euphausia superba*, with incubation period. Line fitted by eye.

there was evidence that the distribution of label between the hepatopancreas and the rest of the body had reached a steady state of about 4 hours (Fig. 2). It was therefore concluded that a 4 hour incubation time was appropriate. Although there were wide individual variations in the amount of label incorporated after this time, the pattern of distribution of label within the body was relatively stable.

The amount of label incorporated per individual krill in all experiments varied from 1001 to 31 746 dpm, equivalent to between 1 % and 32 % of total label introduced to the chamber. Each chamber contained two krill, and the total label incorporated ranged from 1 % to 38 % (median 6 %) of that added.

The wide variation between individuals was most likely due to differences in the number of microcapsules ingested. Differences in feeding activity were apparent during the early stages of the experiment, and microcapsules tended to sink to the bottom of the chamber where they were no longer available to the krill.

Some labelled lipid may have been lost by krill opening the larger microcapsules rather than ingesting them whole. Label in the seawater at the end of the experiment amounted to 18 % of that introduced, in the worst case. This label will have included both [^{14}C]palmitate and $^{14}CO_2$ from catabolized labelled palmitate. These losses, although not negligible, are acceptably small.

Incorporation of [^{14}C]palmitate into total lipid

Two experiments were performed with male krill, each with four individuals. The total activity recovered ranged from 283 to 27368 dpm in hepatopancreas and from 718 to 4378 dpm in muscle, all greater than controls by a factor of at least 45 (Table I). The results of the two experiments were not significantly different, and so the data were combined (Table II). Overall 65% of the ingested label was recovered from the

Table II. Tissue distribution of ^{14}C in lipid of male krill, *Euphausia superba*, fed [^{14}C]palmitate or ^{14}C-labelled algae. Hp, hepatopancreas; Mu, muscle; SE, standard error, *F*, variance ratio for one-way ANOVA of arcsine-transformed data; *P*, two-tailed probability of this result; NS, not significant ($P > 0.05$)

	Percentage of total ^{14}C located in hepatopancreas			dpm Hp/dpm Mu	
	n	*Mean*	*SE*	*Mean*	*SE*
Krill fed [^{14}]palmitate					
Expt 1	4	56.0	9.8	1.57	0.46
2	4	73.4	5.9	3.41	1.04
Combined data	8	64.7	6.2	2.49	0.63
$F_{1,6}$		2.45 (*NS*)		2.64 (*NS*)	
Krill fed ^{14}C-labelled algae					
Expt 1	4	77.1	1.6	3.34	0.37
2	3	75.6	0.5	3.10	0.09
3	2	85.3	2.3	5.97	1.09
Combined data	9	78.4	4.6	3.84	0.47
$F_{2,6}$		9.25 ($P < 0.05$)		8.61 ($P < 0.05$)	

Table III. Tissue distribution of ^{14}C incubation in lipid of mature female krill, *Euphausia superba*, fed [^{14}C]palmitate or ^{14}C-labelled algae. Presentation as for Table II

		Percentage of total ^{14}C located in					
		Hp		*Ovary*		*dpm Hp/dpm Mu*	
	n	*Mean*	*SE*	*Mean*	*SE*	*Mean*	*SE*
Krill fed [^{14}C]palmitate							
Expt 2	4	54.0	11.0	10.6	3.9	1.86	0.57
6	2	69.5	6.9	11.2	2.5	4.93	2.75
Combined data	6	59.1	7.9	10.8	2.6	2.88	1.03
$F_{1,4}$		0.84 (NS)		0.06 (NS)		2.63 (NS)	
Krill fed ^{14}C-labelled algae							
Expt 3	3	71.7	5.2	11.7	3.1	4.36	2.14
4	2	87.9	3.0	5.4	2.0	13.57	2.46
Combined data	5	78.2	5.0	9.2	2.4	8.92	2.39
$F_{1,3}$		6.04 (NS)		2.68 (NS)		5.16 (NS)	

hepatopancreas lipid after 4 hours, and 35% from the rest of the body. The mean ratio of label in hepatopancreas to that in the muscle was 2.5, with 95% confidence limits of 1.2–3.8, which encompass the value of 2.6 at 4 hours found during the time-course experiment.

Two experiments were performed on a total of six mature female krill. These all contained large ovaries and were close to spawning. The recovered label exceeded

control values by a factor of at least 25; again the two experiments were not significantly different and the data were therefore combined (Table III). On average 59% of the ingested label was located in the hepatopancreas, 11% in the ovary and 30% in the muscle. The ratio of label in hepatopancreas to that in muscle was 2.6, similar to that in males.

Table IV. Lipid class distribution of ^{14}C in krill, *Euphausia superba*, fed [^{14}C]palmitate or ^{14}C-labelled algae. PL, phospholipid (in algae these would include glycolipids); TAG, triacylglycerol; Hp, hepatopancreas. Presentation as for Tables I and II

		Percentage of total ^{14}C *located in*			
		PL		*TAG*	
	n	*Mean*	*SE*	*Mean*	*SE*
Krill fed [^{14}C]palmitate					
M Hp	6	42.5	4.0	56.0	4.3
Muscle	6	30.7	5.7	58.8	5.8
F Hp	3	61.7	1.9	32.8	4.0
Ovary	3	29.9	13.0	43.1	12.9
Muscle	3	39.7	7.5	41.4	11.9
Krill fed ^{14}C-labelled algae					
M Hp	6	54.6	1.2	42.8	1.3
Muscle	5	18.5	1.8	80.6	2.0
F Hp	3	54.9	3.0	44.0	3.2
Ovary	3	13.2	1.8	86.8	1.8
Muscle	3	19.5	4.7	80.5	4.7
^{14}C-labelled algae					
Bulk sample	1	61.1	—	27.3	—

Lipid class distribution of ingested label

Although krill were fed [^{14}C]palmitic acid, most of the label was recovered in the form of esterified fatty acid, either as triacyglycerol (TAG) or phospholipid (PL) (Table IV). Free (that is, non-esterified) fatty acid (FFA) is usually present only in small amounts in marine invertebrates. The process of digestion, however, releases quantities of free fatty acid in the gut. Since this FFA is usually rapidly re-esterified once it has traversed the gut wall, it would be expected that in these experiments only a small fraction of the total recovered label would be found in the FFA fraction. This was found to be so; less than 10% of the recovered label was generally found in the wax ester, partial glyceride, FFA and free sterol fractions combined (although in females these components contained 27% of the total label found in the ovary). The amount of ^{14}C recovered from wax ester and partial glyceride was always very low (usually less than 1%) and frequently not distinguishable from background. Although small amounts of wax ester are found in adult *Euphausia superba* (Clarke, 1980) these data suggest that this wax is not metabolically active. It is possible that small amounts of wax are absorbed by krill ingesting copepods and this wax is accumulated without being utilized.

This lipid class distribution of recovered label indicates that in the hepatopancreas and muscle both TAG and PL fractions are being turned over. It is not possible to determine whether the PL fraction is being used in part as a storage lipid, or whether the labelling reflects solely the turnover of membrane lipids.

Experiments with ^{14}C-labelled algae

Some experiments were also performed feeding *Euphausia superba* with algae grown in the presence of [^{14}C]bicarbonate. Whereas in the microcapsule experiment the krill were receiving a full spectrum of marine fatty acids but with only the 16:0 labelled, in these experiments the ^{14}C will have been incorporated into a wide range of fatty acids. Since not all of these fatty acids will have been treated similarly by the metabolic machinery of the krill (polyunsaturated fatty acids, for example, are likely to have been diverted preferentially to phospholipids), the results would be expected to differ from the microcapsule experiments.

This was found to be so. In both male and female *Euphausia superba* the proportion of ingested label incorporated into the hepatopancreas was greater in krill fed ^{14}C-labelled algae than in krill fed [^{14}C]palmitate (Tables II and III). In neither case was the increase quite significant, because of the large individual-to-individual variation within each experiment (ANOVA, both $0.10 > P > 0.05$).

The lipid class distribution of ^{14}C in krill fed labelled algae was strikingly similar in males and females (Table IV). Although in the algae over 60% of the ^{14}C was located in polar lipids, in krill muscle only 20% of the label was located in PL, with 80% in the TAG. In hepatopancreas extracts, 55% of the ^{14}C was in the PL fraction and just under 45% in the TAG. Because of the uncontrolled nature of the ^{14}C labelling in the algae, these results cannot be interpreted any further, and this technique is clearly of limited value in understanding the lipid physiology of herbivorous zooplankton.

Discussion

Previous studies of the fate of [^{14}C]palmitate in large crustaceans (euphausiids, mysiids and decapods) have used a variety of techniques. These have included direct injection of label into the haemocoel (Morris and Sargent, 1973; Kanazawa and others, 1979), allowing the experimental organisms to swim in seawater containing label (Sargent and Lee, 1975), ingestion of label carried on starch granules (Morris and Sargent, 1973; Morris and others, 1973) and assay *in vitro* using tissue fragments (Henderson and others, 1981).

Distribution of incorporated label within the body has been looked at only in *Thysanoessa inermis*. In this species, *in vitro* assay of tissue fragments suggested that incorporation into wax esters was greater in the hepatopancreas than in the abdomen, whereas the reverse was true for triacylglycerol. It is not always easy to interpret *in vitro* assays, though this study showed an excellent agreement between rates *in vitro* and accumulation in the field. However, these data do suggest differing physiological roles for wax ester and triacylglycerol as storage lipid.

Both *Thysanoessa raschii* and *T. inermis* synthesize wax ester and triacylglycerol as storage lipids. They are thus not strictly comparable with *Euphausia superba*, which stores only triacylglycerol. In *T. raschii* exposed to labelled palmitate in seawater, most of the label was incorporated into TAG, with moderate amounts into PL and only small amounts into wax ester (Sargent and Lee, 1975). In contrast, *T. inermis* tissue fragments, incubated *in vitro* with labelled palmitate, tended to incorporate more label into wax ester than TAG (Henderson and others, 1981). Three *Gnathophausia* sp. fed labelled palmitate on starch gave widely varying results from individual to individual (Morris and Sargent, 1973).

Neomysis integer, like *Euphausia superba*, has a lipid composition dominated by TAG and PL. Morris and others (1973) fed *N. integer* with labelled palmitic coated

onto starch, and results from two trials were different. In both lots TAG and PL together contained over 80% of the label incorporated into total lipid, although it is not clear how much of the label in the TAG was due to unesterified FFA. In lot A, TAG + FFA contained 50% of the label and PL 34%, whereas in lot B TAG + FFA contained only 34% and PL contained 62%. Thus in lot A PL was labelled less than neutral lipid, and in lot B PL was labelled more than neutral lipid. The available data thus suggest that the pattern of incorporation varies with species, and probably physiological condition and experimental technique as well.

In the experiments performed here there was a repeatable incorporation of 65% (SE 6%) of the total ingested label in the hepatopancreas in males. In females with maturing ovaries the hepatopancreas contained 59% (SE 8%). The generally repeatable pattern of tissue distribution, and the very high recovery of label as esterified fatty acid indicate that microencapsulated food is a physiologically sensible way of introducing labelled compounds into crustacea. It avoids the damage produced by injection, and the balance of protein to lipid is more realistic than with fatty acid coated on to starch granules.

In all krill examined both triacylglycerol (TAG) and phospholipid (PL) were substantially labelled. The relative strength of incorporation, however, varied between tissues. In both males and females, PL labelling was more intense in hepatopancreas than in muscle. The ratio (dpm in TAG/dpm in PL) differed in the two sexes, and in all cases where it was possible to assess, the amount of palmitate incorporated into TAG was greater than would be expected if all palmitic acid in the krill were turned over at the same rate. This suggests that turnover of TAG depot lipid is faster than turnover of PL, at least as far as palmitic acid is concerned.

In male krill the mean ratio of palmitate incorporated into hepatopancreas to that in the muscle was 2.49 (SE 0.63), close to the value after 4 hours in the time-course study (Fig. 1). When fed labelled algae the ratio was higher (3.84, SE 0.47), but not significantly different overall ($t = 1.75$, 15 *df*, $P > 0.05$). In mature females fed [^{14}C]palmitate the ratio was 2.88 (SE 1.03), not significantly different from males. In mature females fed labelled algae, however, the ratio was significantly higher (8.92, SE 2.39, $t = 2.48$, $P < 0.05$).

The similarity in the distribution of labelled palmitate between hepatopancreas and muscle in males and females suggests that the underlying physiology is also similar. The demand for lipid of the developing ovary may thus be viewed as being superimposed on the normal day-to-day requirements for energy and lipid turnover. Individual variation in the amount of label ingested was too great for any statistically significant difference to be detected between males and females. However female krill fed labelled palmitate incorporated a fairly systematic 10.8% (SE 2.6) of this label into the ovary lipid. For females fed labelled algae the data were similar (9.21%, SE 2.4). Making the assumptions that ovarian demand for energy is superimposed on an unaltered background physiology, and that incorporation of labelled palmitate is representative of the overall demand for energy, these data suggest that energy requirements in a female maturing an ovary are 110% those of a mature male. Neither of these simple assumptions is likely to be strictly true, but an increased food requirement of about 10% is quite reasonable.

The use of microencapsulated foods to introduce radioisotope to crustaceans is clearly a useful technique. In particular, despite a wide variation in the total amount of label incorporated, the tissue distribution and lipid class distribution were very much more repeatable than with previous studies. It is intended to extend the preliminary study described here to investigate the fate of different lipids and fatty acids in *Euphausia superba*, and also euphausiids which synthesize wax ester.

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