TECHNICAL COMMENTS

Demonstration of the Deposition and Modification of Dietary Fatty Acids in Pinniped Blubber Using Radiolabelled Precursors

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ABSTRACT

Radioisotopes are commonly used to study the in vivo metabolism and deposition of dietary fatty acids in adipose tissue. The application of this approach to pinnipeds is problematic because of their large mass and blubber fat content. We have developed a method where labelled lipids can be fed to seals at financially feasible levels, with the radioactivity in individual fatty acids isolated from blubber detected with standard laboratory equipment. A combination of techniques including argentation thin layer chromatography, high performance liquid chromatography with ultraviolet detection, and independent liquid scintillation counting were employed. Juvenile gray seals (*Halichoerus grypus*) were fed either 0.5 mCi ^{3}H-labelled triolein (18:1n-9, $n = 2$) or palmitic acid (16:0, $n = 2$). Blubber samples were taken 12 h later, and the radioactivity in individual fatty acids was determined. Radioactivity was detected in only 18:1 from the animals fed ^{3}H-labelled triolein, indicating direct deposition without modification. Both animals fed ^{3}H-labelled palmitic acid showed clear peaks of radioactivity in 16:0; however, there was also significant activity (23%–29%) found in the desaturation product 16:1. Our results demonstrate that this method is sufficiently sensitive to track the deposition of labelled dietary lipids as well as modification products of ingested fatty acids and will be important in the application of fatty acid signatures to study predator diets.

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Introduction

There is considerable interest in the use of blubber fatty acid composition to study diet in marine mammals, especially pinnipeds (seals and sea lions; Iverson 1993; Käkelä et al. 1993; Iverson et al. 1997*a*, 1997*b*, 2004; Smith et al. 1997; Brown et al. 1999; Kirsch et al. 2000; Walton et al. 2000). Recent work has demonstrated that fatty acid signatures can provide quantitative estimates of predator diets provided that the differential metabolism of individual fatty acids within the predator is accounted for (Iverson et al. 2004). Weighting factors are used to provide a composite picture of the influence of differential deposition, modification, utilization, and de novo synthesis of individual fatty acids on the overall fatty acid composition of blubber. The relative importance of these processes varies with the fatty acid composition and fat content of the diet, potentially altering the values of these weighting factors. The direct investigation of the modification and deposition of specific fatty acids provides insight into the underlying biochemical processes and can be used to understand the effects of variation in diet on these factors.

Desaturation and chain elongation of dietary fatty acids such as 16:0 as well as chain shortening of very long chain monounsaturated fatty acids have previously been suggested to occur in pinnipeds (Ackman et al. 1971; Ackman and Hooper 1974); however, this was based on purely circumstantial evidence. Studying the metabolism and subsequent deposition of fatty acids in vivo requires the use of tracers. Radioisotopes have been used successfully for such studies in small animals such as rats and fish (Owen et al. 1975; Thomassen et al. 1985; Hjelte et al. 1990; Linares and Henderson 1991; Green and Yavin 1993; Rabinowitz and Myerson 1994; Nilsson et al. 1996). Pinnipeds, however, present a significant problem in tracking ingested labelled fatty acids. Pinnipeds are large in body size, and blubber constitutes a high percentage of body mass (approximately 10%–45%; Worthy and Lavigne 1987; Ryg et al. 1990; Markussen and Ryg 1992; Iverson et al. 1995; Arnould et al. 1996; Aarseth et al. 1999; Kirsch et al. 2000); this results in a very large dilution factor for the labelled fatty acid. To ensure that the levels of radioactivity in the adipose samples are above detection limits, mCi amounts of label must be employed rather than the μCi doses typically used with small animals. Although μCi doses have been used in studies involving humans, these studies were not concerned with quantifying the radioactivity

in individual fatty acids but rather just the total radioactivity in an adipose sample (Mårin et al. 1990). When the purpose is to study the fate of specific dietary fatty acids, a proportionately larger dose of radioactivity is required. The cost of such labelled compounds can be quite high. Therefore, studying in vivo lipid metabolism in large animals such as pinnipeds requires the development of methods of analysis that are effective when relatively small doses (<1 mCi) of labelled dietary lipids are used.

We have employed the fatty acid tracer method to study the deposition and potential modification of specific dietary fatty acids in the blubber of juvenile gray seals (*Halichoerus grypus*). Our objectives were to develop a method of analysis that was sufficiently sensitive to detect very small amounts of radioactivity in individual fatty acids in small blubber samples obtained by biopsy sampling and to use this method to determine the fates of two fatty acids common in diets and blubber stores of pinnipeds. This excluded the use of convenient methods normally applied in fatty acid tracer studies such as gas chromatography (GC) or high performance liquid chromatography (HPLC) with direct radioactivity detection, simply because the detection limits of those instruments are much too high to allow the use of an affordable amount of tracer. We instead employed HPLC with UV detection and independent scintillation counting. Because HPLC separation is based on polarity, fatty acid methyl esters (FAME) with differing chain lengths and degrees of unsaturation will coelute. Thus, a preliminary separation of FAME into groups with the same number of double bonds via argentation thin layer chromatography ($AgNO_3$-TLC) was performed.

Material and Methods

Four free-ranging juvenile gray seals were captured on Sable Island, Nova Scotia, placed in a fenced-in, covered enclosure on the beach, and fasted for approximately 12 h. Each seal was fed a diet of approximately 1 kg of ground fish spiked with either 0.5 mCi tritium-labelled triolein ($n = 2$, [9,10-^{3}H(N)]18:1n-9, Mandel Scientific, Ontario) or palmitic acid ($n = 2$, [9,10-^{3}H]16:0, DuPont NEN, Perkin Elmer Life Sciences Canada, Ontario) by gastric intubation. We used only two animals for each study since our main purpose was to develop the analytical methods for tracer feeding and recovery and to validate the identification of modification products. The body composition of the two seals fed ^{3}H-labelled triolein was estimated from measurements of total body water using deuterium dilution methods (Bowen and Iverson 1998) and predictive equations developed for gray seals (Reilly and Fedak 1990). After 12 h, blubber biopsies were taken from both the right and left flank of each animal according to Kirsch et al. (2000). This time period was chosen in light of the rapid rate of passage of digesta in pinnipeds (Helm 1984; Krockenberger and Bryden 1994) and prior evidence of chylomicron clearance from the blood of juvenile gray seals by 12 h postfeeding (Cooper et al. 2003). Blubber obtained from each animal totaled approximately 0.5 g. The animals were then released. Experiments were approved by the Dalhousie University Committee on Laboratory Animals under protocol 98-016.

The decision to feed 0.5 mCi of labelled fatty acid was based on the known detection limit of our scintillation counter and the expected dilution of the ingested label in the animals' blubber. The detection limit of the liquid scintillation counter was approximately 40 dpm, corresponding to 1.8×10^{-8} mCi. Assuming a fat content of 22% (an average value for the age class of gray seals [$n = 31$] used in this study; M. H. Cooper, unpublished data), a 40-kg juvenile gray seal should contain about 8.8 kg of blubber, which would produce an approximately 9,000-fold dilution of the ingested radioactivity on a per gram blubber basis. Under the best case scenario (i.e., all labelled fatty acid is deposited in the blubber), an excess of 3.2×10^{-4} mCi would have to be fed to exceed the detection limit when analyzing 0.5 g of fat. Since one should never plan for the best case scenario, we used an amount 1,000-fold greater than the calculated minimum.

Lipids were extracted from the blubber according to a modified Folch et al. (1957) procedure described in detail by Iverson et al. (2001). Briefly, samples were extracted with 2 : 1 chloroform : methanol and dried over anhydrous sodium sulphate. FAME were formed by reaction of approximately 100 mg of lipid with 1.5 mL of boron trifluoride in methanol (8% v/v) and 1.5 mL of hexane. The mixture was heated at 100°C for 1 h under nitrogen, and FAME were extracted into hexane. FAME samples were separated by degree of unsaturation using $AgNO_3$-TLC (Rezanka 1996). Preparative silica gel plates (250 μm coating thickness, 5 × 20 cm, Sigma Aldrich) were dipped in 20% $AgNO_3$ in acetonitrile and heated at 110°C for 1 h to remove all water. FAME samples (approximately 10 mg) were evaporated to near dryness and spotted on the plate in a single broad band. The band was focused into a narrow line (approximately 1 cm from the base of the plate) by repeatedly developing in 9 : 1 hexane : diethyl ether until the bottom of the band migrated to the top edge of the original band (approximately four repetitions). Plates were dried under a stream of nitrogen between developments. Plates were then fully developed in 9 : 1 hexane : diethyl ether until the solvent front reached the top of the plate. Plates were again dried, sprayed with dichlorofluoroscein, and visualized under UV light. Seven bands were apparent, each representing a group of FAME having the same number of double bonds (from 0 to six). The bands were then scraped from the plate and extracted with 1 : 1 hexane : diethyl ether. GC was used to confirm the purity of each extract.

The FAME of each extract were subjected to reverse phase HPLC equipped with a Supelcosil LC-18 column (30 cm × 4.0 mm inner diameter, 5 μm particle size, Sigma Aldrich) and a 200-μL sample loop. A flow rate of 0.8 mL min^{-1} of 95% aqueous methanol was used for all separations except that of the saturated FAME, for which 98% aqueous methanol was employed (derived from Manku

1983). FAME were detected with UV absorbance at 205 nm. Typically, fatty acids are analyzed as their phenacyl derivatives because these strongly UV-absorbing functional groups increase the detection limits of the instrument (Christie 1982). Our aim, however, was to separate and recover fatty acids with as large a sample throughput as possible, so detecting trace amounts of lipid was not a concern. The use of FAME rather than their phenacyl derivatives also eliminated a step in sample preparation, thus preventing the potential losses associated with additional sample manipulations. Sample throughput was constrained by the column bore, which allowed the injection of only 1 mg FAME peak^{-1}. For a typical fraction containing 10 mg FAME, four to five injections would normally be required. Eluents were manually collected in glass test tubes, and all fractions containing the same individual FAME were pooled. Each pooled fraction was then evaporated under nitrogen until only the water portion remained. Approximately 2 mL of isopropyl alcohol was added, and then both water and alcohol were evaporated. FAME were taken up in hexane and dried over anhydrous sodium sulphate. After GC analysis, each FAME was mixed with scintillation cocktail (ScintiVerse I) and counted in a Beckman Scintillation Counter (LS3801).

GC was used to determine FAME compositions before and after HPLC using a Perkin Elmer Autosystem II Capillary GC equipped with a flame ionisation detector and a flexible fused silica column (30 m × 0.25 mm inner diameter) coated with 50% cyanopropyl polysiloxane (0.25-μm film thickness; J & W DB-23, Folsom, Calif.). Helium (flow rate 1 mL min^{-1}) was used as the carrier gas, and the gas line was equipped with an oxygen scrubber. The following temperature program was employed: 153°C for 2 min, hold at 174°C for 0.2 min after ramping at 2.3°C min^{-1}, and hold at 220°C for 3 min after ramping at 2.5°C min^{-1}. Hydrogen and air had flow rates of 45 and 455 mL min^{-1}, respectively. Both the injector and flame ionisation detector were isothermal at 250°C and 270°C, respectively. Split

injection (100 : 1) with a sample size of 1 μL was employed. FAME were identified by comparison of retention times with known standards (Nu Check Prep, Elysian, Minn.).

Results and Discussion

AgNO$_3$-TLC separation of FAME generated seven bands, with each band predominantly containing FAME of the same degree of unsaturation. With some samples, GC analysis revealed slight contamination (less than 10%) of each band, with FAME having one more or one less double bond than expected. Recoveries from the TLC plate were on average 85% ± 10%, with losses likely occurring when the bands were scraped from the plates and transferred as a powder into the extraction vials containing 1 : 1 hexane : diethyl ether. Despite the presence of potentially interfering FAME of different degrees of unsaturation in each fraction, coelution on HPLC was rarely a problem. Because the interfering peaks were small, in most cases it was possible to collect them as a well-defined shoulder on the larger peaks. Masses of FAME before and after HPLC were very similar with recoveries near 100%, indicating negligible losses at this step.

Figure 1 illustrates the results from the seals fed ^{3}H-labelled triolein (18:1n-9). The 18:1 isolated from blubber clearly contained radioactive tritium, thus verifying that the analytical method proposed here is sensitive enough to track the deposition of labelled dietary lipids. Although the level of radioactivity in 16:1 from the blubber of Hg3416 was slightly above background, it is not clear whether this was analytical noise or a real effect of chain shortening of ^{3}H-labelled 18:1 before deposition. Considering the very small amount of radioactivity found in 16:1, if chain shortening of 18:1 to 16:1 does occur, it likely does not constitute a major pathway in the metabolism of dietary 18:1. Future studies with additional animals and the methods now developed will be important to assess possible between-individual variability in these processes. Figure 2 illustrates the data from the seals fed ^{3}H-labelled palmitic acid (16:0). Both animals exhibited clear peaks of radioactivity in 16:0; in this case, however, there was also significant radioactivity found in 16:1. This is the first direct measure of desaturation of the dietary 16:0 to 16:1 in pinniped blubber and confirms our expectation of the activity of the common Δ9 desaturase enzyme. It also demonstrates that this method is sufficiently sensitive to detect deposition of modification products (16:1) as well as the original ingested fatty acid.

The mass and percent total body fat of the seals fed ^{3}H-labelled triolein are presented in Table 1. Both seals were well within the expected range for percent total body fat but above the average value for their age class of gray seals (22%; M. H. Cooper, unpublished data). However, because body mass was less than 40 kg, the dilution of the radioactive fatty acid was only slightly greater than predicted. The two triolein-fed seals had body fat masses of 11.0 and 9.9 kg (Table 1), corresponding to 11,000- and 9,900-fold dilutions of the ingested radioactivity.
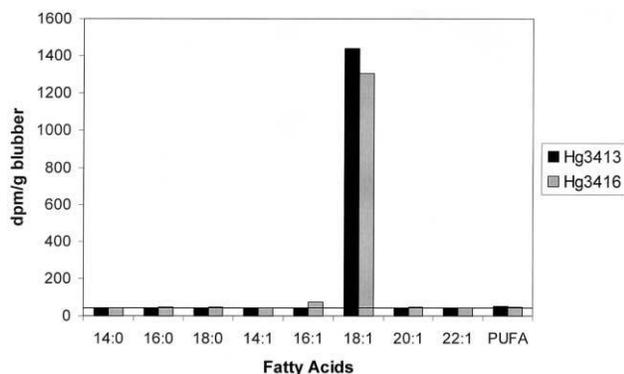

Figure 1. Distribution of radioactivity measured in fatty acids isolated from blubber of each gray seal fed 0.5 mCi of ^{3}H-labelled triolein ([9,10-^{3}H(N)]18:1n-9). Plotted horizontal line represents background radioactivity.
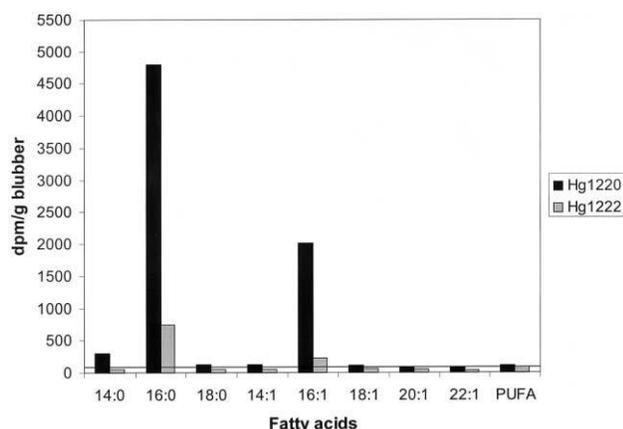
Figure 2. Distribution of radioactivity measured in fatty acids isolated from blubber of each gray seal fed 0.5 mCi of ^{3}H-labelled palmitic acid ([9,10-^{3}H]16:0). Plotted horizontal line represents background radioactivity.

Thus, if all 0.5 mCi of ingested 18:1 was deposited in the blubber, we would expect to see $\geq 4.5 \times 10^{-5}$ mCi g^{-1} blubber or 99,880 dpm g^{-1} blubber. On average, only 1,373.0 $\pm$ 95.3 dpm g^{-1} blubber was found in 18:1. This is equivalent to <2% of the possible maximum.

Because we are confident that approximately 85% of the radioactivity present in the blubber samples is recovered using this technique (calculated from the AgNO$_3$-TLC and HPLC recoveries), this low recovery of radioactivity relative to the possible maximum (<2%) is likely not due to flaws in the chemical method employed. Rather, it appears that the vast majority of labelled fatty acids, ingested with a small meal after 12 h of fasting, are not deposited in the blubber in the first 12 h after ingestion. A major portion of this ingested fat may have been used to fuel immediate metabolic needs, particularly if the labelled lipids are more readily oxidized than natural lipids, which would be in more complex associations with other food components being digested. It is not known whether a larger proportion of radioactivity would have been measured in the blubber had we fed a larger meal, used nonfasted animals, or allowed a different period of time to elapse between ingestion and sampling. Mårin et al. (1990) found that, in humans, the majority of radioactive dietary fatty acid taken up by the adipose tissue was incorporated within the first 24 h, with much of that

being accounted for within the first 4 h. It therefore seems unlikely that sampling later than 12 h postfeeding would have provided any significant improvement in the recovery of radioactivity. Thus, under the current feeding conditions, our use of a quantity of radioactivity 1,000 times in excess of the predicted minimum requirement appears necessary rather than cautious.

The two seals fed ^{3}H-labelled palmitic acid differed dramatically in the amount of radioactivity recovered per gram of blubber sampled, with Hg1220 showing a much stronger radioactive signal in its blubber than Hg1222 (Fig. 2; 4,792.8 vs. 738.5 dpm g^{-1} blubber for 16:0). Unfortunately, body composition was not measured in these animals, so the true dilution of radioactivity into blubber mass is not known. However, on the basis of their mass (Table 1) and the average percent total body fat of 6-mo-old gray seals, Hg1220 was expected to have a radioactivity concentration 1.3 times greater than that found in Hg1222. In actuality, Hg1220 had a concentration 5.7 times greater than Hg1222. Thus, the smaller estimated fat mass and correspondingly smaller dilution of ingested radioactivity in Hg1220 can explain only a small portion of the total difference in radioactivity recovered. It is possible that there was some loss of the labelled fatty acid during the process of gastric intubation. Despite differences in the recoveries of absolute amounts of radioactivity from Hg1220 and Hg1222, the relative proportions of 16:1 produced from 16:0 were similar (29% and 23% of ^{3}H recovered, respectively). These values may be of use in the understanding and refinement of weighting factors for 16:0 and 16:1 used in estimating diets based on fatty acid signatures (Iverson et al. 2004).

Our use of independent scintillation counting was justified on the basis of the levels of radioactivity recovered in the blubber. With the pooling of fractions containing the same individual fatty acids, the maximum level of radioactivity detected in any one fatty acid was approximately 7,000 dpm g^{-1} FAME for 16:0 in Hg1220. Because the HPLC column capacity was 1 mg FAME peak^{-1}, the maximum level of radioactivity that could have been detected had this HPLC been equipped with a scintillation counting device was ~7 dpm. This is below the detection limit of radioflow detectors, which typically have background levels around 10 dpm, confirming the impracticality of such equipment for these purposes. Therefore, this method of analysis improves the level of detection by approx-

Table 1: Mass and body fat measurements of individual study animals

Seal	Labelled Lipid	Mass (kg)	Percent Total Body Fat	Fat (kg)
Hg3413	[9,10-^{3}H(N)]triolein	37.0	29.8	11.0
Hg3416	[9,10-^{3}H(N)]triolein	38.0	26.1	9.9
Hg1220	[9,10-^{3}H]palmitic acid	31.5	…	…
Hg1222	[9,10-^{3}H]palmitic acid	41.5	…	…

imately three orders of magnitude. This will be particularly important for future studies using other labelled fatty acids that can be as much as five to six times more expensive than those used in this study.

The AgNO₃-TLC-HPLC procedure described here is labour intensive as a result of the multiple HPLC injections of the bands recovered from the TLC plate. An obvious solution is computer-automated sample injection and fraction collection, allowing analysis to continue without human intervention. A second possibility is prescreening of samples for radioactivity after AgNO₃-TLC separation. It is clear from Figures 1 and 2 that the polyunsaturated fatty acid fractions were not radioactive. Had a small portion of these fractions been prescreened, this absence of radioactivity could have been established before the HPLC analysis, saving much time and labour. Clearly, however, the combination of AgNO₃-TLC-HPLC with independent scintillation counting allows the appearance of radioactively labelled fatty acids to be accurately tracked in the blubber of juvenile gray seals. Specifically, this method makes the in vivo study of the metabolism of individual fatty acids in large mammals financially feasible by allowing a reasonably small amount of label to be employed. In addition, we have provided the first direct evidence in pinnipeds of the deposition without modification of a monounsaturated fatty acid (18:1n-9) as well as the importance of the Δ9 desaturase enzyme to the metabolic fate of a dietary saturated fatty acid (16:0). The results of this study and those of future studies of other important dietary fatty acids will provide information pertinent to the quantitative application of fatty acid signatures to estimate diets.

Acknowledgments

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