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Carbon-by-carbon discrimination of ^{13}C incorporation into liver fatty acids

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Quantitative carbon-by-carbon analysis would be useful in determining the origin and fate of carbons involved in fatty acid metabolism. Incorporation of ^{13}C from 2-[^{13}C]acetate into specific carbons of liver fatty acids was lowest at the n–2 carbon of saturates and monoenes but was 47% greater at acyl C1 than at C2, suggesting substantial redistribution of the ^{13}C from C2 to C1 of acetyl CoA or malonyl CoA prior to ^{13}C incorporation into fatty acids during de novo synthesis or during elongation. Thus, ^{13}C derived from exogenous acetate can be quantitatively measured and is differentially incorporated into individual carbons depending on position in the fatty acid molecule.

Fatty acid; [^{13}C]Acetate; ^{13}C NMR; Liver

1. INTRODUCTION

Carbon-13 nuclear magnetic resonance spectroscopy (^{13}C NMR) is a useful tool in metabolic studies because of the low natural abundance of ^{13}C and because ^{13}C NMR signals of individual carbons of extracted compounds in solution are widely dispersed and usually well-resolved permitting accurate integration of peak areas [1–7]. Several individual fatty acid carbons can be clearly distinguished by ^{13}C NMR permitting determination of the percent composition of the four major classes as well as some individual long chain fatty acids [8–10]. ^{13}C NMR would also permit analysis of ^{13}C incorporation using ^{13}C-labelled fatty acid substrates but application of this methodology to studies of fatty acid synthesis and metabolism has not been reported. However, the one-step sample preparation and carbon-specific data suggests it could be very useful in assessing step-by-step synthesis and degradation of long chain fatty acids under a variety of conditions.

2. MATERIALS AND METHODS

Protocols approved by the Animal Care Committee, Faculty of Medicine, University of Toronto were used for all procedures involving animals. 99% 2-[^{13}C]sodium acetate (MSD Isotopes, Montreal, Quebec) was dissolved in 2.0 ml distilled water and injected i.p. (0.75 mg/kg body weight; 100–125 g male Wistar rats). Controls were injected with saline. The rats were killed 24 h post-injection. Total liver lipids were quantitatively extracted into 20 volumes of chloroform/methanol (2:1, v/v), dried under nitrogen gas and weighed. A known amount of the lipid extract (80–100 mg) was redissolved in 500 μl deuterated chloroform (Silanor; MSD Isotopes, Montreal) containing 10 mg of the internal standard - tetrakis [trimethylsilyl] silane (TMSS; Aldrich Chemical Co. Milwaukee, WI) and transferred to a 5 mm NMR tube (Wilmad Glass Co., Buena, NJ).

^{1}H decoupled ^{13}C NMR spectra were obtained using a Bruker AM300 WB spectrometer (Bruker Canada, Milton, Ontario). The ^{13}C free induction decay of each sample was acquired at 75.4 MHz using a sweep width of 17,241 Hz and 16K data points. Other acquisition parameters were: signal acquisition time, 0.475 s; relaxation delay, 10 s; excitation pulse, 30 degrees. Each spectrum comprised 5,000 sequentially-added transients and took about 15 h to acquire. Free induction decays were down-loaded from the spectrometer to an IBM-AT type computer for Fourier transformation and peak integrations (Marquardt-Levenberg routine; NMR 286 program, Softpulse Software, Guelph, Ontario). Integrals for ^{13}C peak areas were compared with that of TMSS. Total fatty acids and fatty acid classes were determined as previously described [8]. The method was shown to be linear over a sample weight range of 20–150 mg and intrasample variation was 1–5% (larger peak areas had smaller variation) [12].

3. RESULTS AND DISCUSSION

The composition (% and mg/liver) of fatty acid classes in the rat liver fatty acid extracts agreed well between ^{13}C NMR and capillary gas liquid chromatography (Table I). Total fatty acids/liver were quantitated using the integral of the terminal methyl carbon (14.0–14.1 ppm). Thus, under the present spectrometer operating conditions, ^{13}C NMR provides reliable values for % and absolute composition of the four major fatty acid classes containing 16–22 carbons in rat liver lipid extracts.

24 h after injection of 99% 2-[^{13}C]sodium acetate, ^{13}C enrichment at several fatty acid carbons was evident (Fig. 1). Amongst discrete, single carbon resonances, relative ^{13}C enrichment was greatest at C1 and decreased towards the methyl terminal of fatty acids

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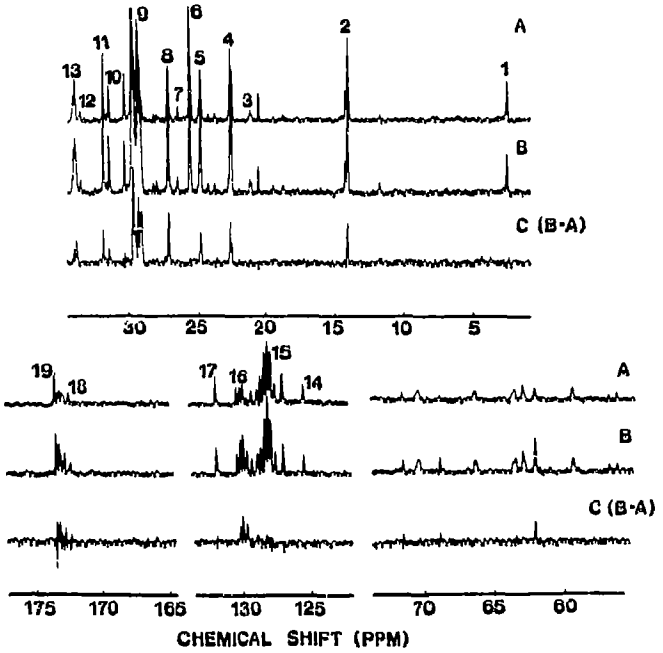

Fig. 1. Natural abundance ^{13}C NMR spectrum of a chloroform/methanol extract of rat liver total lipids containing only natural abundance ^{13}C (A) compared to a spectrum of rat liver total lipids 24 h after enrichment with ^{13}C from 2-[^{13}C]acetate (B). The difference spectrum (C) illustrates the specific carbons at which ^{13}C enrichment has occurred. The numbered peaks are: (1) 2.61 ppm, TMSS; (2) 14.04–14.20 ppm, Sum of n−1 carbons; (3) 20.51 ppm, n−2 carbons (n−3 polyenes); (4) 22.53–22.64 ppm, n−2 carbons (saturates, monoenes and n−6 polyenes) + C3 (22:6n−3); (5) 24.52–24.91 ppm, sum of C3 carbons except 22:6n−3; (6) 25.59 ppm, doubly allylic carbons (n−6 and n−3 polyenes); (7) 26.44 ppm, C4 carbon (20:4n−6, 20:5n−3); (8) 27.17 ppm, allylic carbons (monoenes, n−6 and n−3 polyenes except 22:6n−3); (9) 29.05–29.64 ppm, sum aliphatic carbons; (10) 31.49 ppm, n−3 (n−6 polyenes); (11) 31.89 ppm, n−3 carbons (saturates and monoenes); (12) 33.39 ppm, C2 carbon (20:4n−6, 20:5n−3); (13) 33.95–34.05 ppm, sum C2 carbons (except 20:4n−6, 20:5n−3); (14) 127.00 ppm, n−4 carbon (n−3 polyenes); (15) 127.52–128.97 ppm, 'inner' olefinic carbons; (16) 129.44–130.42 ppm, 'outer' olefinic carbons; (17) 131.96 ppm, n−3 carbon (n−3 polyenes); (18) 174.06 ppm, C1 carbon (22:6n−3); (19) 174.33–174.38 ppm, Sum C1 carbons (except 22:6n−3).

(Table II). Odd-numbered carbons, particularly the n-2 carbon (peak 4 in Fig. 1), of both saturates and monoenes had the lowest ^{13}C enrichmetn. The high ^{13}C enrichment at C1 indicates recycling of the ^{13}C from C2 of acetate to C1 of acetyl or malonyl CoA prior to fatty acid incorporation. ^{13}C enrichment at C1 was dispersed between several different fatty acids and at different 'sn' positions in the extracted liver lipids; their exact identification requires higher magnetic strength for resolution. The lower relative and actual ^{13}C enrichment at n-1 than at C1 suggests that the number of fatty acid molecules partially labelled with ^{13}C exceeded net ^{13}C incorporation into fatty acids. Hence, as well as providing the total carbon skeleton for some fatty acids, the exogenous ^{13}C also contributed to elongation of fatty acids already partially synthesized.

Table I

Comparison of percent and actual amounts (mg/liver) of fatty acid classes in rat liver total lipid extracts as determined by quantitative ^{13}C NMR spectroscopy (NMR) versus weighing plus capillary gas liquid chromatography (GLC) (n=4; mean ± S.D.). Coefficient of correlation between percent composition data for the two methods was 0.994 (P<0.0001; n=32 data points).

	NMR	GLC
% Composition		
Sum *cis* n−6 polyenes	29.4 ± 1.3	29.9 ± 2.1
Sum *cis* n−3 polyenes	7.6 ± 1.2	7.3 ± 0.9
Sum *cis*-monoenes	17.3 ± 1.0	16.0 ± 2.3
Sum saturates	45.7 ± 3.1	46.8 ± 3.1
Absolute composition (mg/liver)		
Sum *cis* n−6 polyenes	8.6 ± 1.7	11.6 ± 1.5
Sum *cis* n−3 polyenes	2.2 ± 0.6	2.8 ± 0.3
Sum *cis*-monoenes	5.8 ± 1.0	6.1 ± 0.8
Sum saturates	18.2 ± 2.8	18.0 ± 2.3
Sum total fatty acids	34.9 ± 5.2	38.5 ± 5.0

Amongst multiple carbon resonances, ^{13}C enrichment was greatest at 'outer' than at 'inner' olefinic carbons (peaks 16 and 15 in Fig. 1, respectively); monoenes contain only 'outer' olefinic carbons whereas polyenes contain both 'outer' and 'inner' olefinic carbons. Thus, from the ^{13}C enrichment at olefinic carbons, synthesis of monoenes (mainly palmitoleic acid and oleic acid) considerably exceeded that of polyenes (mainly arachidonic acid and docosahexaenoic acid since linoleic acid cannot be synthesized by animals). The low synthesis of arachidonic or docosahexaenoic acids was con-

Table II

Relative and actual ^{13}C enrichment in specific carbons of rat liver fatty acids 24 h after i.p. injection of 99% 2-[^{13}C]sodium acetate. Peak numbers are identified in Fig. 1. Natural abundance values are those obtained from a control rat (A in Fig. 1) while the ^{13}C enrichment values are those from a [^{13}C]acetate-injected rat (B in Fig. 1).

Peak number	Assignment*	Natural abundance (mg/liver)	^{13}C enrichment	
			%	(μg/liver)
Single carbon resonances				
2	n−1#	1.62	+ 55	+ 9.91
4	n−2	0.68	+ 34	+ 2.57
11	n−3	0.92	+ 76	+ 7.78
19	C1##	0.95	+145	+15.32
13	C2	1.16	+ 81	+10.45
5	C3	1.10	+ 71	+ 8.69
Multiple carbon resonances				
8	Allylic	1.95	+ 16	+ 3.47
9	Aliphatic	9.42	+ 55	+57.62
15	Olefinic ('inner')	3.74	+ 18	+ 7.49
16	Olefinic ('outer')	0.39	+197	+ 8.55

*see Fig. 1 (legend) for complete information
#'n' refers to the methyl terminal of the fatty acid
##'C' refers to the carboxyl terminal of the fatty acid

firmed by the virtual absence of ^{13}C enrichment at doubly allylic carbons (peak 6 in Fig. 1). Although polyenes and monoenes have both 'outer' olefinic and allylic carbons, only one allylic carbon in polyenes but two in monoenes can become ^{13}C labelled in animals. ^{13}C enrichment at allylic carbons was <10% of that at 'outer' olefinic carbons, suggesting there may be some differentiation between ^{12}C and ^{13}C at this carbon position.

This methodology, which requires minimal sample preparation, indicates that absolute changes in ^{13}C mass as well as relative ^{13}C enrichment in fatty acid extracts of animal tissues can be readily discriminated at individual fatty acid carbons in complex lipid mixtures. Being able to distinguish the actual amount and ^{13}C enrichment of each fatty acid class, as well as carbon-by-carbon ^{13}C enrichment within fatty acid classes should have application in studies of fatty acid synthesis and metabolism.

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