Positional Distribution of Fatty Acids in Triglycerides from Milk of Several Species of Mammals

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ABSTRACT

Milk triglycerides from the echidna, koala, Tammar wallaby, guinea pig, dog, cat, Weddell seal, horse, pig and cow were subjected to fatty acid and stereospecific analysis to determine the positional distribution of the fatty acids in the triglycerides. The samples presented a wide range of fatty acids, most of which varied in content among species. The compositions of the acids at the 3 positions also varied among species, reflecting the content of these acids in the triglycerides. However, there was a general similarity in fatty acid positional distribution patterns for all the species with the exception of the echidna. The echidna exhibited a completely different fatty acid positional distribution pattern. The saturated acids were preferentially esterified at the *sn*-1-position whereas the unsaturated acids were selectively esterified at the *sn*-2-position. The triglyceride carbon number distribution of milk from the above species (with the exception of the Weddell seal) was determined by gas liquid chromatography and compared to that predicted by the 1-random-2-random-3-random fatty acid distribution hypothesis. Agreement was excellent between observed and predicted composition for echidna, koala, Tammar wallaby, guinea pig and pig milk, and agreement was reasonable for dog, cat, horse and cow milk. Results are discussed in relation to biochemical mechanisms.
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Milk triglycerides are synthesized from fatty acids derived from plasma triglycerides (TG) and from de novo synthesis in the mammary gland (1). While the *sn*-glycerol-3-phosphate pathway is considered to be the major synthetic pathway, others, such as the monoglyceride pathway, may also make a significant contribution (2). The positional distribution of fatty acids in milk triglycerides has been determined for the cow (3), sheep (4), goat (4), human (5), pig (6) and rat (7). These studies indicate that milk triglycerides are asymmetrical with the short-chain fatty acids preferentially esterified at the *sn*-3-position.

To determine if the positional distribution of fatty acids in milk triglycerides from all species followed a uniform pattern, 8 other species, representing 5 different orders, were studied. The pig and cow, species which have been examined previously, were included in the study. The TG carbon number distribution of the milks was compared with that calculated from the 1-random-2-random-3-random fatty acid distribution hypothesis.

MATERIALS AND METHODS

Samples

Milk samples were obtained from the koala (*Phascolarctus vinereus*), guinea pig (*Cavia porcellus*), dog (*Canis familiaris*), cat (*Felis domesticus*), Weddell seal (*Leptonychotes weddelli*), horse (*Equus caballus*), pig (*Sus scrofa*) and cow (*Bos taurus*). Lipids were extracted with diethyl ether and petroleum ether (boiling range 30-60 C) by the Roese-Gottlieb method (8). Milk lipids from the echidna (*Tachyglossus aculeatus*) and Tammar wallaby (*Macropus eugenii*) were extracted from milk using chloroform and methanol by CSIRO, Division of Wildlife Research. Samples were stored under nitrogen at -20 C until they were required for analysis. Triglycerides were obtained from milk lipid by column chromatography using 7% hydrated Florisil (9).

Stereospecific Analysis

The *sn*-1,2(2,3)-diglyceride method of Brockerhoff (10), adapted for mg quantities by Christie and Moore (11) was used with modification. This method, together with the pancreatic lipase deacylation procedure used to obtain monoglycerides, was reported by Parodi (12). In the current study, diglycerides for stereospecific analysis were generated by the Grignard reagent, ethyl magnesium bromide. In the case of cow milk, diglycerides were obtained using a pancreatic lipase deacylation. A sample of interesterified cow milk fat was used to obtain optimal conditions for pancreatic lipase deacylation. Results for the *sn*-1-position were obtained by analysis of the lysophosphatide, those for the *sn*-2-position were obtained from monoglycerides by pancreatic lipase deacylation and those for the *sn*-3-position were calculated by difference from the known triglyceride composition. The composition of the *sn*-2,3-diacyl-1-phosphatidyl phenols pro-

vided a check for the *sn*-3-position.

Fatty Acid and Triglyceride Analysis

Triglycerides and partial glycerides were transesterified to methyl esters (13). Phospholipids were transesterified by the addition of 5 μl of 2.0 N methanolic sodium methoxide and 50 μl of hexane. Methyl esters and TG carbon number distribution were analyzed by gas liquid chromatography (GLC) as previously reported (12,14).

RESULTS

Triglyceride fatty acid composition and the positional distribution of the fatty acids in the triglycerides from the milk of the 10 species of mammals is given in Table 1. The milk that was studied presented a wide range of fatty acids, most of which varied in content among species. The compositions of the acids at the 3 positions also varied among species, reflecting the content of these acids in the triglycerides. However, there was a general similarity in fatty acid positional distribution patterns for all the species with the exception of the echidna. The 4:0 and 6:0 acids were exclusively esterified at the *sn*-3-position whereas 8:0 was preferentially esterified at this position. For the horse, 10:0 was selectively associated with the *sn*-3-position buf, in the cow, there was slightly more of this acid at the *sn*-2-position than at the *sn*-3-position. The 12:0, 14:0 and 16:0 acids were preferentially associated with the *sn*-2-position except in the cow, where there was a little more 16:0 at the *sn*-1-position than at the *sn*-2-position. In all species, 18:0 was selectively esterified at the *sn*-1-position. For the seal and horse, 18:1 was preferentially associated with the *sn*-1-position but, for the other species, it was preferentially associated with the *sn*-3-position. The 18:2 and 18:3 acids were always preferentially esterified at the *sn*-3-position.

The echidna exhibited a completely different fatty acid positional distribution pattern. The 14:0, 16:0 and 18:0 acids were preferentially esterified at the *sn*-1-position whereas the unsaturated acids 18:1, 18:2 and 18:3 were selectively esterified at the *sn*-2-position.

A computer program was devised to calculate the amounts of the triglycerides predicted by the 1-random-2-random-3-random fatty acid distribution hypothesis. The program also allowed for the predicted triglycerides to be summed into groups according to their carbon number. Data from the stereospecific analysis of echidna, koala, Tammar wallaby, guinea pig, dog, cat, horse, pig and cow milk triglycerides were used to generate the TG carbon number distribution predicted by the 1-random-2-random-3-random fatty acid distribution hypothesis. This was compared to carbon number distribution determined experimentally by GLC. The difference between the observed and calculated composition for each carbon number distribution was determined. As the specificity in utilization of particular fatty acids in certain triglycerides must be balanced by discrimination in others, the sum of either the positive or negative differences (D mol %) was used to test deviation from the 1-random-2-random-3-random fatty acid distribution hypothesis.

Results for the 9 species of mammals are presented in Table 2. Agreement was excellent between TG carbon number distribution, calculated by the 1-random-2-random-3-random fatty acid distribution hypothesis, and that determined experimentally for echidna, koala, Tammar wallaby, guinea pig and pig milk. For these animals, the value of D was less than 5 mol %. It was difficult to assess the effect of experimental error from TG carbon number distribution and stereospecific analyses on the value of D. A subjective estimate considered that D values above 5 mol % may indicate deviation from the distribution hypothesis. Dog, cat, horse and cow milk had D values between 5 and 7.5 mol % and it may be considered that there was reasonable agreement for TG carbon number distribution determined experimentally and that calculated from the 1-random-2-random-3-random hypothesis.

The major triglycerides in most milk samples were C 50, C 52 and C 54. In general, C 54 was present in amounts greater than that predicted by the fatty acid distribution hypothesis whereas the C 52 and C 50 triglycerides occurred in less-than-predicted amounts.

DISCUSSION

The species of mammals selected for study provided a wide range of fatty acid types and compositions for a comparison of fatty acid positional distributions in triglycerides. There has been a number of stereospecific analyses of bovine milk triglycerides from butter or individual cows (3,12,15-17). The results show that 4:0 and 6:0 are always almost exclusively esterified at the *sn*-3-position. The 12:0 and 14:0 acids are always preferentially esterified at the *sn*-2-position whereas 18:0 is always preferentially associated with the *sn*-1-position. In some samples, 18:1 is selectively esterified at the *sn*-1-position and in others at the *sn*-3-position. The 8:0 and 10:0 acids are selectively esterified

TABLE 1

Positional Distribution of Fatty Acids in Triglycerides from Milk of Several Mammals

Species	Position	Fatty acid composition (mol %)																
		4:0	6:0	8:0	10:0	12:0	14:0	14:1	15:0	15:1	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3	Other
Echidna	TG	–	–	–	–	–	1.0	0.7	0.4	0.2	22.8	7.4	1.1	0.7	11.1	43.5	9.1	2.0	–
	sn-1	–	–	–	–	–	1.7	1.3	0.8	0.4	31.5	7.1	1.5	0.7	16.8	33.1	4.1	1.0	–
	sn-2	–	–	–	–	–	0.9	0.7	0.2	0.1	9.0	7.0	0.4	0.8	2.1	57.6	18.3	2.9	–
	sn-3	–	–	–	–	–	0.4	0.2	0.1	0.2	27.9	8.0	1.6	0.6	14.3	39.8	4.9	2.0	–
Koala	TG	–	–	–	–	0.1	3.9	0.2	0.8	–	25.7	4.6	1.1	0.8	4.9	16.1	10.3	31.5	–
	sn-1	–	–	–	–	0.1	3.9	0.2	1.0	–	25.3	5.8	2.5	1.2	14.4	20.7	7.4	20.5	–
	sn-2	–	–	–	–	0.1	8.6	0.3	1.6	–	50.4	6.1	0.7	0.6	1.0	4.8	5.3	20.5	–
	sn-3	–	–	–	–	0.1	-0.7	–	0.1	–	1.5	1.8	0.2	0.6	2.2	22.7	18.1	53.4	–
Wallaby	TG	–	–	–	0.1	0.1	1.7	0.2	0.5	0.1	19.6	6.0	0.6	0.7	2.7	51.6	8.8	7.3	–
	sn-1	–	–	–	0.1	0.2	1.9	0.4	0.7	0.2	21.2	6.1	0.8	0.7	5.3	52.8	6.6	3.0	–
	sn-2	–	–	–	0.1	0.3	3.8	0.3	0.7	0.2	34.8	9.9	0.8	0.9	2.3	33.3	6.6	6.0	–
	sn-3	–	–	–	–	–	-0.6	–	–	–	3.0	2.1	0.1	0.6	0.4	68.4	13.1	12.9	–
Guinea pig	TG	–	–	–	–	0.1	2.5	0.2	0.3	0.1	30.9	3.0	0.6	0.5	2.8	33.8	20.3	4.9	–
	sn-1	–	–	–	–	–	2.7	0.2	0.5	0.1	24.1	4.1	1.0	0.6	6.9	40.8	15.6	3.4	–
	sn-2	–	–	–	–	0.1	4.6	0.3	0.5	0.1	68.1	3.7	0.6	0.4	0.6	7.3	11.6	2.1	–
	sn-3	–	–	–	–	–	0.2	–	–	–	0.4	1.3	0.2	0.6	1.0	53.3	33.9	9.1	–
Dog	TG	–	–	–	0.2	0.7	4.9	1.1	0.7	0.2	26.5	7.8	0.9	1.1	3.7	40.9	9.2	2.1	–
	sn-1	–	–	–	0.1	0.3	3.4	0.6	0.9	0.2	34.5	7.3	1.2	0.9	6.3	39.2	4.8	0.4	–
	sn-2	–	–	–	0.2	1.1	10.3	1.7	1.1	0.3	42.6	9.8	0.8	1.0	1.2	20.1	8.8	1.0	–
	sn-3	–	–	–	0.3	0.6	1.0	1.1	–	0.1	2.4	6.5	0.7	1.5	3.6	63.4	14.1	4.7	–
Cat	TG	–	–	–	0.4	0.9	5.4	0.9	0.8	0.2	26.8	5.1	1.2	0.8	10.1	40.3	5.8	1.3	–
	sn-1	–	–	–	0.1	0.3	2.8	1.1	0.7	0.2	20.5	4.8	1.4	0.7	16.4	45.5	4.4	1.1	–
	sn-2	–	–	–	0.4	1.5	12.4	0.9	1.8	0.3	51.1	6.3	1.1	0.7	2.4	15.5	4.6	1.0	–
	sn-3	–	–	–	0.7	1.0	0.9	0.7	-0.2	0.1	8.9	4.1	1.1	1.0	11.7	59.5	8.6	1.9	–
Seal	TG	–	–	–	–	0.2	11.5	1.3	0.4	0.2	15.0	13.7	0.5	0.9	2.1	39.5	2.1	0.5	12.1
	sn-1	–	–	–	–	0.2	7.3	1.9	0.6	0.1	13.1	10.2	0.3	0.8	4.5	53.8	1.3	0.3	5.6
	sn-2	–	–	–	–	0.3	23.6	1.9	1.1	0.3	31.0	16.8	0.6	0.7	0.7	19.4	2.3	0.5	0.8
	sn-3	–	–	–	–	0.2	3.8	0.1	0.1	0.3	1.0	14.1	0.6	1.2	1.0	45.4	2.8	0.7	28.7
Horse	TG	–	0.3	2.3	3.6	4.3	5.0	0.9	0.3	0.2	16.4	9.0	0.1	0.5	1.0	15.2	9.3	29.8	1.8
	sn-1	–	–	0.2	0.7	3.0	5.1	1.1	0.6	0.2	23.5	8.4	0.4	0.5	2.7	20.6	7.2	25.6	0.2
	sn-2	–	–	3.0	4.6	6.3	9.0	1.1	0.7	0.2	25.4	10.9	0.1	0.5	0.6	7.5	7.2	20.1	2.8
	sn-3	–	0.9	3.6	5.5	3.7	0.8	0.5	-0.2	0.1	0.2	7.6	-0.1	0.4	-0.3	17.4	13.6	43.7	2.6
Pig	TG	–	–	–	–	0.1	2.4	ND[a]	0.2	ND	26.9	4.0	0.6	ND	6.8	30.3	27.3	1.4	–
	sn-1	–	–	–	–	0.1	1.8	ND	0.3	ND	26.4	3.5	0.8	ND	11.2	32.7	21.8	1.4	–
	sn-2	–	–	–	–	0.2	5.1	ND	0.5	ND	48.1	5.1	0.5	ND	1.8	15.5	21.9	1.2	–
	sn-3	–	–	–	–	-0.1	0.4	ND	-0.2	ND	6.2	3.4	0.5	ND	7.5	42.8	38.0	1.6	–

Cow[b]	TG	8.8	5.0	2.5	5.3	5.2	13.8	1.7	1.0	0.2	28.0	2.3	0.6	0.2	8.3	14.1	1.1	0.9	1.0
	sn-1	–	–	0.2	1.4	3.4	12.7	1.4	1.5	0.3	41.2	2.7	1.0	0.3	15.2	16.2	1.2	0.7	0.6
	sn-2	–	0.1	2.9	7.8	8.1	23.9	2.4	1.8	0.4	36.7	3.3	0.5	0.2	3.0	7.2	0.6	0.3	0.8
	sn-3	26.4	14.8	4.4	6.7	4.0	4.9	1.2	-0.3	–	6.1	1.0	0.2	0.2	6.7	19.0	1.6	1.7	1.4

[a]Not determined.

[b]The 14:1, 15:1, 16:1 and 17:1 acids also contain 15:0 br, 16:0 br, 17:0 br and 18:0 br, respectively; 18:3 also contains 18:2*c*,*t* conj.

TABLE 2

Observed Triglyceride Carbon Number Distribution Composition and the Composition Calculated Using the 1-Random-2-random-3-random Fatty Acid Distribution Hypothesis for Milk from 9 Species of Mammals

Carbon number	Composition (mol %)																	
	Echidna		Koala		Wallaby		Guinea pig		Dog		Cat		Horse		Pig		Cow	
	Obs[a]	Calcd[b]	Obs	Calcd	Obs	Calcd	Obs	Calcd	Obs	Calcd	Obs	Calcd	Obs	Calcd	Obs	Calcd	Obs	Calcd
24	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.0	0.1
26	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.4	0.3
28	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.9	1.1
30	–	–	–	–	–	–	–	–	–	–	–	–	0.1	0.1	–	–	1.7	2.6
32	–	–	–	–	–	–	–	–	–	–	–	–	0.3	0.2	–	–	3.8	4.7
34	–	–	–	–	–	–	–	–	–	–	–	–	0.7	0.4	–	–	8.4	8.4
36	–	–	–	–	–	–	–	–	–	–	–	–	1.7	0.9	–	–	13.3	12.5
38	–	–	–	–	–	–	–	–	–	–	–	–	2.9	1.6	–	–	13.6	12.0
40	–	–	–	–	–	–	–	–	0.0	0.1	0.1	0.1	3.7	2.8	–	–	10.1	8.3
42	–	–	–	–	–	–	–	–	0.2	0.2	0.4	0.4	5.8	5.3	–	–	8.3	6.8
44	–	–	0.0	0.1	0.1	0.1	0.2	0.0	1.2	0.9	1.6	1.2	8.4	8.7	–	–	7.6	7.2
46	0.6	0.7	1.2	0.9	1.0	0.6	1.3	0.4	4.3	3.2	4.3	3.5	9.4	10.3	1.2	0.6	7.5	8.1
48	5.0	5.6	7.0	7.1	4.6	3.9	6.2	4.9	12.2	12.5	10.8	11.6	10.3	11.2	6.0	4.9	8.0	10.1
50	22.7	23.6	27.4	27.9	19.7	19.5	25.0	26.8	27.8	32.5	24.6	29.8	15.4	18.2	22.1	23.8	8.4	10.6
52	41.1	42.9	45.5	45.1	42.6	44.8	53.0	53.9	34.9	37.1	38.9	40.4	24.7	25.5	43.4	46.2	5.8	6.0
54	30.6	27.2	18.9	18.9	32.0	31.1	14.3	14.0	19.4	13.5	19.3	13.0	16.6	14.8	27.3	24.5	2.3	1.2
D(mol %)	3.4		0.7		2.1		2.7		7.3		7.5		5.7		4.5		7.2	

[a]Observed.

[b]Calculated.

at either the *sn*-2- or the *sn*-3-position. In many samples, there is little difference in fatty acid composition at the 2 positions competing for preferential esterification. The interspecies differences in fatty acid positional distribution noted in Table 1 are of the same nature as the intraspecies differences noted for the cow.

Stereospecific analysis of triglycerides from the sheep and goat (4,18), human (5) and rat (7) show that these species also exhibit a similar fatty acid distribution pattern to that just outlined. In human and rat triglycerides, 12:0 is preferentially esterified at the *sn*-3-position rather than at the *sn*-2-position.

Factors which influence the specific fatty acid distribution pattern in milk triglycerides include: acyl-CoA concentrations, acyltransferase specificity and activity and the biochemical pathways used for triglyceride synthesis. Studies with the bovine (19-22) and with the rat (7,23,24) demonstrate that the fatty acid specificities of mammary *sn*-glycerol-3-phosphate acyltransferases and 1-acyl-*sn*-glycerol-3-phosphate acyltransferases are related to the fatty acid composition at the *sn*-1- and *sn*-2-positions of milk triglycerides. The fatty acid composition at the *sn*-3-position is not determined to any great extent by the specificities of bovine mammary diacylglycerol acyltransferases (25).

Gross and Kinsella (20) and Kinsella (21) found that the specific activity of palmitoyl-CoA:*sn*-glycerol-3-phosphate acyltransferase from the mammary tissue of different cows varied widely. It can be assumed that the activities of other acyltransferases will also vary among animals and this may explain why a particular fatty acid is preferentially esterified at different positions in some samples.

It is now generally accepted that milk triglycerides are mainly synthesized by the *sn*-glycerol-3-phosphate pathway, however, in the pig, Bickerstaffe and Annison (2) showed that a monoglyceride pathway was as active as the *sn*-glycerol-3-phosphate pathway. The positional distribution of fatty acids in pig milk triglycerides reported in Table 1 and by Christie and Moore (6) is similar to other species, including the cow, in which the *sn*-glycerol-3-phosphate pathway is known to be the major, if not the only, synthetic pathway. The *sn*-glycerol-3-phosphate pathway is also the major pathway for adipose tissue triglyceride synthesis (26). These triglycerides have a fatty acid distribution pattern different from milk triglycerides, indicating that other factors such as acyltransferase specificity are more important than the synthetic pathway in determining fatty acid distribution.

The positional distribution of fatty acids in echidna milk triglycerides is different from the distribution in other species. Grigor (27) also has recently shown, by pancreatic lipase deacylation, that the proportional distribution of fatty acids at the *sn*-2-position of milk triglycerides from this species is different from other animals. His results are comparable to data in Table 1. The echidna, along with the platypus, is the most primitive surviving mammal. Although the structure of monotreme mammary glands is similar to those of marsupials and eutherians (28), the glands may be unspecialized (29).

Of the various animal tissues, the depot fat of mammals most closely resembles echidna milk triglycerides in the positional distribution of fatty acids (30). However, the symmetrical nature of echidna milk triglycerides is more akin to those of common vegetable oils (31). Tissue and organ microsomal fractions may contain *sn*-glycerol-3-phosphate and 1-acyl-*sn*-glycerol-3-phosphate acyltransferases with different fatty acid specificities from those of the mammary gland (24). For the diacylglycerol acyltransferases, this may not be the case (25). It is thus possible that echidna mammary tissue contains acyltransferases with different specificities than those of other mammals, although other factors may be involved. Stokes and Tove (32) presented evidence that pig adipose tissue contained a specifier factor which, by interacting with acyltransferases, appeared to direct the acylation of 16:0 to the *sn*-2-position.

Most past studies, as reviewed by Litchfield (33), used triglyceride class composition to evaluate 1,3-random-2-random or 1-random-2-random-3-random fatty acid distribution hypotheses. Although this approach allows for the chain length of the fatty acids, it does not distinguish between type and degree of unsaturation. Recently, Managanaro et al. (34) have applied a more sophisticated approach to determining enantiomeric structures of peanut oil triglycerides. Using chromatographic techniques and stereospecific analysis detailed analyses of the molecular species of generated *sn*-1,2-, *sn*-2,3- and *sn*-1,3-diglycerides led these workers to conclude that the fatty acids in the 3 positions of the glycerol molecule were combined with each other solely on the basis of their relative molar concentrations. As a result, it was possible to calculate the composition of the molecular species of the peanut oil triglycerides using the 1-random-2-random-3-random hypothesis.

For pig milk, Christie and Moore (6) found excellent agreement between triglyceride class composition and that calculated by a 1-random-

2-random-3-random distribution hypothesis. In the current study, agreement was also excellent for pig milk when TG carbon number distribution was compared to that expected by the 1-random-2-random-3-random hypothesis. In Table 2, the milks with the highest D values were those with the largest range of fatty acids (Table 1). The magnitude of D in these milks may be related to specificity due to the chain length of the fatty acids.

Biochemical evidence to support a 1-random-2-random-3-random fatty acid distribution in milk triglycerides is very limited. For the bovine, Marshall and Knudsen (22) found that the chain-length specificity of the acyltransferases was unaffected by the nature of the fatty acid (palmitic or oleic acid) at the *sn*-1-position of 1-acyl-*sn*-glycerol-3-phosphate. This is an example of noncorrelative acylation (35). Lin et al. (7) found, with lactating rat mammary gland, that acyl-CoA specificity was affected by the type of 1,2-diglyceride acceptor offered. However, although dilaurin was the best acceptor and *sn*-1,2-dilaurin > *sn*-1,2-dimyristin > *sn*-1,2-dipalmitin > *sn*-1,2-distearin, the authors could not say unequivocally that the apparent preference for shorter-chain diglycerides did not result at least partially from the greater solubility of these substrates.

TG carbon number distribution is a necessary, put perhaps insufficient, test of the 1-random-2-random-3-random fatty acid distribution hypothesis. Although care must be exercised when using the hypothesis to calculate triglyceride composition, the procedure does provide information quickly. This information would perhaps take years to obtain using other analytical techniques. Kuksis (36), however, has cautioned that obtaining data from distribution hypotheses should not stifle the development of analytical procedures which will provide such data without making a priori hypotheses.

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