Quantitative gas–liquid chromatographic analysis of rodent milk triglycerides

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ABSTRACT A comparison has been made of the milk and adipose tissue triglycerides of rabbits and guinea pigs provided with one diet and of rats and mice provided with another. Both intact triglycerides and component fatty acids were analyzed by gas–liquid chromatography. Good correlation of the data obtained by the two techniques was obtained by calculating the average chain length of the fatty acid moieties.

Little difference was found in the triglyceride composition of the adipose tissue of the different species. However, wide variation in the triglyceride composition of the milk was found between the species: the average fatty acid chain length in milk was 11.7 for rabbits, 14.2 for rats, 15.3 for mice, and 17.2 for guinea pigs. The corresponding values for adipose tissue were in the range 16.9–17.4 in all animals. The significance of enzymes that synthesize short-chain fatty acids in mammary gland is discussed.

KEY WORDS gas–liquid chromatography · triglycerides · fatty acids · rodent · milk · adipose tissue · diet

ALTHOUGH SOME INFORMATION (1–6) is available on the fatty acid composition of rodent milk, little is known of the composition of the intact triglycerides, which we have therefore analyzed by GLC.

Much of our present knowledge of the biosynthesis of milk fats has been derived from studies on rodent mammary tissue (7–12), but few workers have related results obtained in vitro to the fatty acid composition of the milk of the species under investigation.

Abbreviations and terminology: GLC, gas–liquid chromatography; C_4–C_{18}, fatty acids with 4 to 18 carbon atoms; C_{24}–C_{56}, triglycerides with a total number of acyl carbon atoms of 26 to 56; 8-8-8 and 8-10-8, etc. refer to triglycerides with three C_8, or two C_8 and one C_{10} fatty acids per molecule, respectively.

Note. Rabbits are included here in the order of Rodentia, though they may be considered as a distinct order, the Lagomorpha.

Our previous work with extracts from the mammary gland of lactating rabbits has concerned the pathways of synthesis for fatty acids of various chain lengths (13). The present study was initiated in an attempt to correlate these findings with the actual fatty acids present in rabbit milk triglycerides.

MATERIALS

Methyl ester standards were made from the free fatty acids (>99% pure, Fluka AG, Basel, Switzerland) by the method of Metcalfe and Schmidtz (14). Triglyceride standards were those described in the previous paper (15). Oxytocin was supplied by Sandoz Ltd., Basel, Switzerland.

A sample of pasteurized cow's whole milk was purchased from Midland Counties Dairies Ltd., Birmingham, England. The sample of human milk was kindly donated by a mother who was at the end of her lactation period (about 2 months). Albino rabbits (5–15 days post partum) 1 and 2 were New Zealand white strain; rabbit 3 was a colored animal of unknown strain (see Tables). Guinea pigs (5–10 days post partum) were piebald and random bred in the departmental colony. Albino mice (12 days post partum) were taken from a closed colony, in which the mice had been randomly bred for 15 yr (departmental Vale strain). Albino rats (14 days post partum) were from a closed colony (departmental Vale strain), in which the rats had been randomly bred for 15 yr; this colony was originally started with rats from the Wistar Institute.

Mixed crushed oats and Diet 18 rabbit pellets were supplied by Morning Foods, Crewe, England. Heygate (Modified Diet 41B) rat cubes were from Bugbrook Mills, Bugbrook, Northampton, England.

Rabbits and guinea pigs were provided with a diet of mixed crushed oats, Diet 18 rabbit pellets, and cabbage.

Rats and mice were provided with a diet of Heygate (Modified Diet 41B) rat cubes. All animals were given an adequate supply of water. As far as can be ascertained, these diets had not been substantially altered since the colonies were started.

GLC materials and equipment are described in the accompanying paper (15). For GLC of methyl esters, polyethylene glycol adipate and Apiezon L, both on Chromosorb W (hexamethyl disilazane treated), 100–120 mesh, were purchased from Applied Science Laboratories Inc., State College, Pa., as was boron trifluoride–methanol reagent. Fluorescent MN-Silica Gel/UV_{254} was supplied by Mackerey, Nagel & Co., Düren, Germany. Reagents were of analytical grade when available.

METHODS

Lactating rats, guinea pigs, and mice were given an intraperitoneal injection of oxytocin (0.4 IU/kg) 20 min before they were killed by cervical dislocation. Milk was removed from the mammary glands (care being taken to avoid contamination by blood) and the lipids were immediately extracted. Rabbits were killed without prior injection of oxytocin and the mammary glands were dissected out and placed on an ice-cold surface. An adequate supply of milk exuded from the glands under these conditions.

Adipose tissue from the back of rats, guinea pigs, and rabbits was dissected out, but the mesentery was the source of the adipose tissue from mice.

Adipose tissue was homogenized in chloroform–methanol 2:1 with 20 ml of solvent per g of tissue in an MSE Atomix (purchased from Measuring and Scientific Equipment, London, England) at top speed for 30 sec. Milk was vigorously shaken for 30 sec with 2 × 20 volumes of chloroform–methanol 2:1. All lipid extracts were purified by the procedure of Folch, Lees, and Sloane Stanley (16).

Samples containing significant amounts of lipid other than triglycerides were purified by thick-layer chromatography: 20 × 20 cm chromatoplates were coated with a 1 mm layer of fluorescent Silica Gel G. 50 mg of lipid in *n*-hexane was applied as a streak to each plate and developed in diethyl ether–*n*-hexane 40:60. No sample was found to contain more than 1% of cholesteryl ester.

Triglyceride samples were taken up in *n*-hexane and the solution was stored in the presence of anhydrous sodium sulfate. A further precaution was to store the stoppered flasks of samples in a desiccator containing CaO. Intact triglycerides were analyzed by GLC immediately after extraction and purification. Samples stored for methanolysis were found to be stable for at least 3 wk when stored dry, but were normally analyzed within 1 wk. Sample deterioration could be readily detected by changes in the pattern of intact triglycerides determined by GLC. Methanolysis was carried out as described by Luddy, Barford, and Riemenschneider (17), with sodium methoxide prepared from magnesium-dried methanol; the product mixture was applied directly to the column. To confirm completion of methanolysis, we subjected the mixtures produced from cow's milk triglycerides to GLC under conditions appropriate to intact glycerides (15). No peaks corresponding to triglycerides were obtained.

Dietary fatty acids were obtained by saponification of samples of the diets with ethanolic NaOH under reflux for 8 hr, filtration of the mixture through glass wool, acidification to pH 2 with concentrated hydrochloric acid, and extraction of the free acids with *n*-hexane. The hexane extracts were combined and dried. The methyl esters were prepared with boron trifluoride–methanol (14). GLC of the methyl esters of fatty acids was carried on a Pye Panchromatograph equipped with 152 × 0.65 cm columns of 10% polyethylene glycol adipate or 10% Apiezon L. The esters were detected in an argon ionization chamber at a flow rate of 60 ml/min. Analyses on polyethylene glycol adipate employed a temperature program of 75–175°C at 6°C/min: those on Apiezon L were isothermal at 197°C. While chromatography on polyethylene glycol adipate separated mono-, di-, and triunsaturated fatty acids, Apiezon L separateed saturated and monounsaturated acids and the combination of the results enabled each sample to be completely analyzed. Each fatty acid was quantified by comparison with methyl ester standards. Detector response was linear for each methyl ester over the range of chain lengths used. Molar correction factors were obtained for each methyl ester quoted. Peak areas were measured by triangulation.

The compositions of intact triglycerides were determined on a 10% SE-30 column (15). A typical separation of rabbit milk triglycerides is illustrated in Fig. 1.

All samples were analyzed in duplicate on each column used. Molar correction factors were obtained with the aid of standard mixtures [these data are presented in the accompanying paper (15)]. Values obtained from duplicate analyses showed absolute deviations of ±2% for major components (>5% of total) and ±5% for minor components (< 5% of total). Unidentified, odd-numbered, and minor (< 0.1 mole %) but identified fatty acids were ignored. Odd-numbered trigylcerides were ignored, as well as even-numbered triglycerides of < 0.1 mole %.

The average fatty acid chain length was calculated by the following formulae: for fatty acid methyl esters, $C_a = {}^1/_{100}\ \Sigma_n\ (C \times \text{moles \%})$; for triglycerides, $C_a = {}^1/_{100}\ \Sigma_n\ (\mathbf{C}/3 \times \text{moles \%})$, where C = fatty acid carbon number, $\mathbf{C}$ = triglyceride carbon number, and n = number of components in mixture.

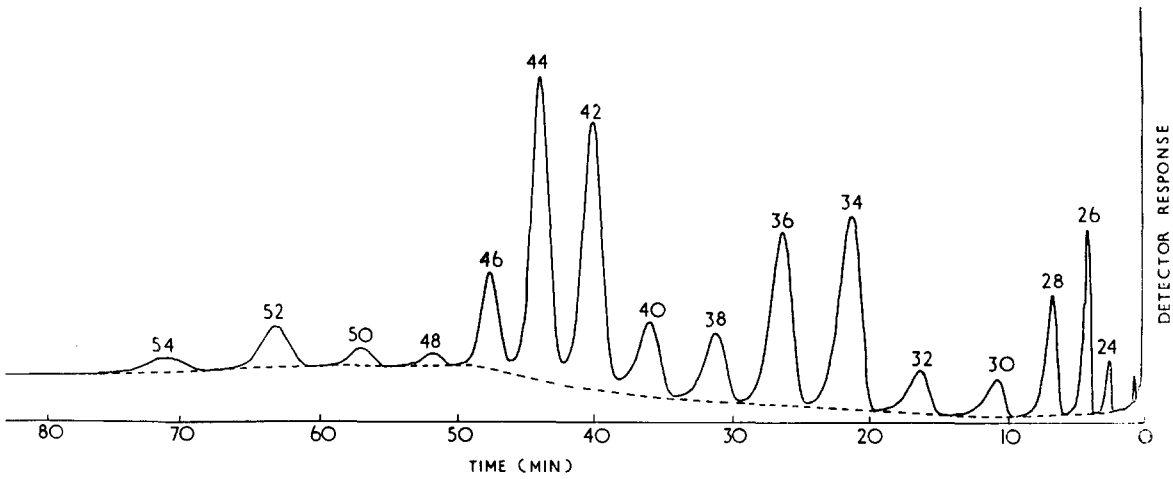

FIG. 1. Programmed-temperature GLC of intact triglycerides of rabbit milk fat. The triglycerides are denoted by total acyl carbon atoms. The dotted line represents the base line. For experimental details, see Methods.

RESULTS AND DISCUSSION

The most significant feature of previous studies (18) was that the milk fats of land mammals, particularly herbivores, differed from the corresponding depot fats in that they contained significant amounts of short-chain fatty acids.

Our study of a small group of rodents (cf Tables 1 and 2) has confirmed this trend in rabbit, rat, and mouse, but not in guinea pig. With respect to the fatty acid composition of milk, short-chain fatty acids accounted for about 65% of the total acids in rabbit, but only 0.1% in guinea pig (Table 1). This difference was also reflected in the composition of the intact milk triglycerides (Table 3). Triglycerides of acyl carbon number less than 42 made up about 75% of the total triglycerides in rabbit milk, whereas guinea-pig milk contained no significant amounts of such triglycerides. Furthermore the triglycerides 8-8-8, 8-10-8, and 10-8-10 which make up approximately 30% of the total, appear to be confined to the milk fat of rabbit. These strikingly different results appear to represent the extremes of milk triglyceride composition so far reported, despite the fact that both species were provided with the same diet (oats, pellets, and cabbage). However, we did not determine the absolute amounts of fat in the pellets, oats, and cabbage or the proportions of these three components that were eaten.

Studies on rats and mice also revealed marked differences in milk fatty acid and intact triglyceride composition (cf Tables 1 and 3), although these species were both provided with Heygate rat cubes. Short-chain fatty acids were observed to contribute about 24% of the total fatty acids in rat milk but only about 8% of the total in mouse milk (Table 1). Similarly, 45% of the rat milk triglycerides contained less than 42 carbon atoms, but only 20% of mouse milk triglycerides fell into this category (Table 3).

TABLE 1 FATTY ACID COMPOSITION OF RODENT MILK FATS (MOLES %)

Fatty Acid (No. of Carbon Atoms: No. of Double Bonds)	Rabbit			Guinea Pig			Mouse			Rat	
	1	2	3	1	2	3	1	2	3	1	2
4:0	—	—	—	—	—	—	—	—	—	—	—
6:0	0.7	0.2	0.6	—	—	—	—	—	—	0.4	0.9
8:0	44.9	32.8	33.6	—	—	—	0.2	0.5	0.4	7.6	4.2
10:0	23.4	40.7	18.2	0.1	0.1	—	6.1	8.3	8.0	20.3	13.7
12:0	1.1	5.8	1.7	0.1	0.1	—	8.7	13.2	13.8	13.0	12.3
14:0	0.8	1.4	1.0	1.9	2.5	1.3	13.1	16.4	17.9	10.2	14.6
16:0	8.6	4.2	11.3	36.4	30.2	36.7	32.9	33.1	31.8	26.3	29.6
16:1	0.9	0.4	1.1	1.6	2.3	1.0	5.1	2.7	3.3	1.6	2.0
18:0	1.4	1.3	2.3	4.2	3.7	1.9	2.2	1.8	1.5	3.3	2.0
18:1	8.4	5.3	14.0	30.0	31.3	31.7	22.3	15.7	15.5	12.6	12.6
18:2	9.3	7.3	14.6	22.3	24.4	23.3	9.4	8.3	7.9	4.8	8.2
18:3	0.5	0.6	1.7	3.3	5.5	4.1	—	—	—	—	—
Average Fatty Acid Carbon Number (C_a)	11.3	11.0	12.7	17.2	17.2	17.2	15.1	15.1	15.7	13.8	14.4

Samples of milk from individual animals were used. In all Tables — for a result represents <0.1 mole % present.

TABLE 2 FATTY ACID COMPOSITION OF RODENT DEPOT FATS (MOLES %)

Fatty Acid (No. of Carbon Atoms: No. of Double Bonds)	Rabbit		Guinea Pig		Mouse		Rat	
	1	3	1	2	2	3	2	3
10:0	—	—	—	—	0.1	—	—	—
12:0	—	—	—	—	0.2	0.2	—	0.3
14:0	3.5	3.0	1.0	1.6	2.4	2.1	1.4	2.0
16:0	43.3	28.1	27.7	28.0	26.0	26.4	31.1	31.6
16:1	3.3	4.1	1.6	1.4	7.9	6.5	5.7	6.3
18:0	6.6	7.7	8.2	8.3	6.0	5.5	2.8	2.7
18:1	20.9	27.0	28.4	28.6	39.1	43.1	31.2	31.8
18:2	22.4	28.3	29.2	27.2	18.4	16.2	27.9	25.3
18:3	—	1.7	4.0	5.0	—	—	—	—
Average Fatty Acid Carbon Number (C_a)	16.9	17.2	17.4	17.4	17.2	17.2	17.2	17.2

Samples were from the same animals as in Table 1.

TABLE 3 TRIGLYCERIDE COMPOSITION OF RODENT MILK FATS (MOLES %)

Triglyceride (Carbon Number)	Rabbit			Guinea Pig			Mouse			Rat	
	1	2	3	1	2	3	1	2	3	1	2
22	—	0.2	—	—	—	—	—	—	—	—	—
24	3.5	1.8	1.2	—	—	—	—	—	—	0.1	—
26	14.8	10.6	7.3	—	—	—	—	—	—	0.7	0.6
28	11.5	15.0	5.6	—	—	—	—	—	—	1.8	0.3
30	5.9	12.1	2.5	—	—	—	—	—	—	4.6	1.1
32	6.8	8.6	2.9	—	—	—	0.3	0.7	0.8	7.2	2.0
34	15.5	12.1	15.0	—	—	—	0.6	1.2	1.6	9.2	3.7
36	11.5	12.8	12.7	—	—	—	1.5	2.5	3.1	9.5	5.8
38	4.8	7.8	4.8	—	—	—	3.1	5.4	6.4	10.2	8.8
40	5.0	4.6	4.6	—	—	—	6.3	8.9	10.3	10.3	12.3
42	9.9	5.7	14.9	0.5	0.4	—	8.7	13.2	14.4	9.8	14.5
44	7.2	5.5	15.3	0.6	0.6	—	10.6	14.3	15.4	7.9	14.6
46	1.1	2.2	5.0	1.5	1.5	0.5	11.2	14.0	13.8	6.6	12.0
48	0.4	0.4	0.7	4.9	4.5	3.4	12.3	12.0	12.8	7.2	8.8
50	0.8	0.4	1.5	25.9	22.7	23.4	15.5	11.7	10.6	7.4	7.7
52	1.2	0.3	4.2	59.7	60.4	63.6	23.2	12.4	8.6	6.2	6.0
54	0.3	—	1.8	6.9	9.9	9.0	6.2	3.7	2.2	1.2	1.8
56	—	—	—	—	—	—	0.5	—	—	—	—
Average Fatty Acid Carbon Number (C_a)	11.4	11.2	12.8	17.1	17.1	17.2	15.8	15.2	14.9	13.6	14.3

Samples are the same as those used in Table 1.

In terms of degree of unsaturation of the milk triglycerides, guinea pig was outstanding in that only 40% of the total acids were saturated compared with 80% for rabbit and rat, and 60% for mouse (Table 1). The ratio of mono- to diunsaturated acids was lower in rabbits (0.8–1.0) and guinea pigs (1.4) than in rats (1.8–2.9) and mice (2.3–2.9) (Table 1). The lower proportion of diunsaturated fatty acids in this latter group was perhaps a little surprising in view of the particularly high content of linoleic acid in the diet (Table 4).

The average fatty acid chain length (C_a) calculated from the milk triglycerides and fatty acid data showed good agreement (cf Tables 1 and 3). Although some variation in average fatty acid chain length occurred within a species, there was no overlap between species.

Analysis of the triglycerides from adipose tissue of each species (Tables 2 and 5) revealed no specific differences in

TABLE 4 FATTY ACID COMPOSITION OF DIETARY MATERIAL (MOLES %)

Fatty Acid (No. of Carbon Atoms: No. of Double Bonds)	Rabbits and Guinea Pigs			Rats and Mice
	Pellets	Oats	Cabbage	Rat Cubes
14:0	0.7	2.4	—	1.0
16:0	23.8	26.2	14.4	16.2
16:1	0.8	—	5.1	0.9
18:0	3.8	32.4	3.2	3.4
18:1	14.6	—	—	13.9
18:2	47.8	39.0	22.7	59.9
18:3	8.4	—	54.5	4.8

average fatty acid chain length. The presence of significant amounts of linolenic acid in guinea pig (Table 2) and one of the rabbit adipose tissue samples (Table 2), and the absence of this acid from the adipose tissues of rat and mouse (Table 2) may result from the inclusion of cab-

bage in the diet of the guinea pig and rabbit (Table 4). The agreement between average chain-length data obtained from fatty acid and glyceride data was again good (Tables 2 and 5).

We have been unable to find any comparable data on rodent milk compositions in the literature except in the case of rat. Rees, Shuck, and Ackermann (6) found only about 6% of fatty acids of chain length less than 12 in rat milk, which compares with about 24% in our case (Table 1). This may be a consequence of the different diets offered to the animals, by Rees and ourselves. In neither case did the diet contain significant amounts of short-chain fatty acids. In spite of the differences in dietary fatty acid composition, the rats used by Rees et al. (6) and ourselves had rather similar adipose composition. Litchfield, Harlow, and Reiser (19) have reported the triglyceride and fatty acid composition of rat adipose tissue, but found a very different fatty acid composition from those reported here. The reason for this is not clear, as dietary data were not reported. However, the major triglyceride detected in both investigations is C_{52} (C_{16}, di-C_{18}).

A report (5) on the fatty acid composition of guinea-pig milk stated that it contained 9.2% (w/w) butyric acid. We have been unable to substantiate this result and in fact, found no trace of butyric acid. The value of GLC of intact triglyceride to confirm a fatty acid analysis is well illustrated by this example. No triglyceride below C_{42} was observed (Table 3). This excludes any possibility of significant (>0.1%) amounts of butyric acid, since the maximum acyl carbon number expected of a butyro-triglyceride would be 40 (two C_{18} and one C_4 moieties).

The only other extensive work, using GLC of intact glyceride, has been that of Kuksis, McCarthy, and Beveridge on butter fat (20). For comparison, we have analyzed the triglycerides of cow's and human milk (Table 6). The percentage composition of cow's milk triglyceride was in close agreement with that obtained by Kuksis et al. (20), as was the average fatty acid chain length. It is interesting that although the average fatty acid chain length of cow's milk triglyceride falls within the range found for rat and mouse milk (cf Tables 1 and 3), the percentage compositions of triglycerides and fatty acids show distinct differences (cf Tables 1, 3, and 6). This is mainly because of the higher proportion of C_4 and C_{18} acids in cow's milk (Table 6).

Data obtained from a single sample of milk from a woman in late lactation are shown also in Table 6. The fatty acid composition is similar to previously reported results (21). The most notable difference from cow's (Table 6), rabbit, and rat milk (Tables 1 and 3) is the absence of short-chain fatty acids and triglycerides from human milk.

TABLE 5 TRIGLYCERIDE COMPOSITION OF RODENT DEPOT FATS (MOLES %)

Triglyceride (Carbon Number)	Rabbit		Guinea Pig		Mouse		Rat	
	1	3	1	2	2	3	2	3
42	0.2	0.4	0.2	0.2	0.3	0.2	0.2	0.1
44	0.5	0.6	0.3	0.3	0.7	0.6	0.3	0.2
46	3.8	2.4	0.8	0.9	1.4	1.4	1.1	0.8
48	16.8	9.9	4.6	6.0	6.3	6.9	6.4	5.6
50	36.4	25.8	17.7	23.0	23.7	25.1	25.7	24.9
52	28.4	39.0	40.4	42.0	40.7	39.3	42.7	43.4
54	13.4	20.3	34.6	26.8	25.8	25.5	21.9	24.1
56	0.6	1.7	1.6	1.1	1.3	1.1	1.8	1.1
Average Fatty Acid Carbon Number (C_a)	16.9	17.1	17.4	17.3	17.2	17.2	17.2	17.3

Samples are the same as those used in Table 2.

TABLE 6 FATTY ACID AND TRIGLYCERIDE COMPOSITION OF HUMAN AND COW'S MILK (MOLES %)

Species	Average Fatty Acid Carbon Number	Fatty Acid (No. of Carbon Atoms: No. of Double Bonds)										
		4:0	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2
Human	16.3	—	—	0.4	2.4	8.9	8.8	25.8	2.7	5.9	35.2	9.9
Cow	14.6	10.6	3.1	1.4	2.8	3.0	12.7	25.7		39.0		1.7

		Triglyceride (Carbon Number)																
		24	26	28	30	32	34	36	38	40	42	44	46	48	50	52	54	56
Human	16.4	—	—	—	—	—	—	0.5	1.0	2.0	3.9	7.6	11.3	13.5	19.5	32.6	7.7	0.6
Cow	14.6	0.1	0.3	0.8	1.2	2.3	4.8	9.4	13.2	11.3	5.8	4.9	6.0	8.5	12.5	12.5	6.5	—

When short-chain fatty acids are absent from diet and adipose tissue triglyceride, but present in characteristic amounts in the milk (for example in the rabbit, Table 1), the presence in the mammary gland of enzyme systems that synthesize these acids is indicated. Since rabbits and guinea pigs were provided with the same diet, it would be interesting to study fatty acid biosynthesis in vitro in the guinea-pig mammary gland in view of the almost complete absence of short-chain fatty acids from the milk.

Studies in vitro (13) on the mammary gland of lactating rabbit have demonstrated its ability to synthesize fatty acids of chain length 6–18 via the malonyl CoA pathway. In addition Singh and Kumar (22) and Smith and Dils (13) have also shown that butyric acid can be synthesized via a pathway that does not require malonyl CoA. The absence of butyric acid from rabbit milk triglycerides (Table 1) throws some doubt on the importance of this pathway in vivo.

The results presented show the value of the GLC of intact triglycerides as an additional tool in mammalian lipid analysis. They are not intended as a detailed examination of the orgin of specific tissue triglycerides, which is currently being investigated.

At the time of writing our attention has been drawn to a paper by Breckenridge and Kuksis (23). This paper presents data for triglyceride compositions of the milks from several species, including guinea pig. Our findings for the triglyceride composition of guinea pig milk are in good agreement with those presented by Breckenridge and Kuksis.

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References

1. Luckey, T. D., T. J. Mende, and J. Pleasants. 1954. *J. Nutr.* **54:** 345.
2. Jart, A., J. P. Funch, and H. Dam. 1959. *Acta Chem. Scand.* **13:** 1910.
3. Beare, J. L., E. R. W. Gregory, D. M. Smith, and J. A. Campbell. 1961. *Can. J. Biochem. Physiol.* **39:** 195.
4. Beare, J. L. 1961. *Can. J. Biochem. Physiol.* **39:** 1855.
5. Baker, B. E., E. I. Bertok, and A. I. Symes. 1963. *Can. J. Zool.* **41:** 1041.
6. Rees, E. D., A. E. Shuck, and H. Ackermann. 1966. *J. Lipid Res.* **7:** 396.
7. Popják, G., and M. L. Beeckmans. 1950. *Biochem. J.* **46:** 547.
8. Hele, P., G. Popják, and M. Lauryssens. 1957. *Biochem. J.* **65:** 348.
9. Hele, P., and G. Popják. 1958. *Proc. Roy. Soc. (London) Ser. B*, **149:** 414.
10. Hele, P. 1958. *Brit. Med. Bull.* **14:** 201.
11. Abraham, S., K. J. Matthes, and I. L. Chaikoff. 1961. *Biochim. Biophys. Acta.* **49:** 268.
12. Dils, R., and G. Popják. 1962. *Biochem. J.* **83:** 41.
13. Smith, S., and R. Dils. 1966. *Biochim. Biophys. Acta.* **116:** 23. 1966. *Biochim. Biophys. Acta.* **125:** 435.
14. Metcalfe, L. D., and A. A. Schmidtz. 1961. *Anal. Chem.* **33:** 363.
15. Watts, R., and R. Dils. 1968. *J. Lipid Res.* **9:** 40.
16. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. *J. Biol. Chem.* **226:** 497.
17. Luddy, F. E., R. A. Barford, and R. W. Riemenschneider. 1960. *J. Am. Oil Chemists' Soc.* **37:** 447.
18. Garton, G. A. 1963. *J. Lipid Res.* **4:** 237.
19. Litchfield, C., R. D. Harlow, and R. Reiser. 1965. *J. Am. Oil Chemists' Soc.* **42:** 849.
20. Kuksis, A., M. J. McCarthy, and J. M. R. Beveridge. 1963. *J. Am. Oil Chemists' Soc.* **40:** 530.
21. Insull, W., Jr., and E. H. Ahrens, Jr. 1959. *Biochem. J.* **72:** 27.
22. Singh, V. N., and S. Kumar. 1963. *Biochim. Biophys. Acta.* **70:** 711.
23. Breckenridge, W. C., and A. Kuksis. 1967. *J. Lipid Res.* **8:** 473.