Molecular weight distributions of milk fat triglycerides from seven species

W. C. BRECKENRIDGE and A. KUKSIS

Banting and Best Department of Medical Research, University of Toronto, Toronto 5, Canada

ABSTRACT The triglyceride compositions of the milk fats of man, dog, guinea pig, cow, sheep, goat, and horse were compared by gas–liquid chromatography of the intact triglycerides and of the butyl esters of the component fatty acids. The milk fats of man, dog, and guinea pig, which were largely made up of long-chain fatty acids, showed a common pattern with major contributions made by the glycerides with 48–54 acyl carbon atoms. The milk fats of cow, sheep, and goat, which were rich in short-chain acids, showed significant proportions of triglycerides with 28–54 acyl carbon atoms. Horse milk, which contains large amounts of medium-chain fatty acids, gave a characteristic triglyceride pattern in the 26–54 carbon atoms range. The experimentally determined distributions of the molecular weights of the triglycerides of all milk fats deviated significantly from the distributions predicted by random association of the fatty acids from a single pool. The data suggest that in all species the milk fat may be formed by a partial resynthesis of preformed glycerides.

KEY WORDS triglycerides · gas–liquid chromatography · milk fat · man · dog · guinea pig · cow · sheep · goat · horse · butyl esters of fatty acids · random distribution

THE COMPOSITION of milk triglycerides has been frequently examined for indications of the mechanism of their biosynthesis. Since the invention of GLC, detailed analyses of the fatty acid content of the milk triglycerides of various species have become available (1–3). In several instances the positional distribution of the fatty acids in the glyceride molecules has also been determined (4). In all cases a preferential esterification has been noted, for at least some of the component acids, a either the primary or the secondary position, which suggested some degree of nonrandomness during the process of milk fat synthesis.

Reports on GLC analyses of intact triglycerides of milk fats have been limited to the cow (5) and goat (6). The pooled samples of butterfat examined (5) indicated a nonrandom distribution of the glycerides on the basis of molecular weight. The composition of the goat milk triglycerides was not quantitatively evaluated. This report provides in detail the molecular weight distributions of the milk fat triglycerides of seven species and compares the data to the appropriate random predictions. The milks of man, cow, goat, and horse were selected for their importance in human nutrition. Those of guinea pig and dog and that of the sheep were included because of their similarity in fatty acid composition to the milk fats of man and cow, respectively.

MATERIALS AND METHODS

Standard monoacid triglycerides (C_{24}–C_{54}) of 99% purity were purchased from the Applied Science Laboratories Inc., State College, Pa. All solvents and reagents were of Fisher Certified Reagent grade quality.

Milk Samples

The milk was obtained from individual mature and healthy females 2–4 wk after parturition. Single samples were collected from horse, sheep, goat, and dog, duplicate samples from guinea pig, and multiple samples from man and cow. The samples varied in size from 1–2 ml (guinea pig) to 10–50 ml (dog, horse, goat, and sheep) and 100–250 ml (man and cow). All the animal milks were collected in the fall (October–November). The sheep, horse, and goat were grazing or received hay. The cows were grazing and received a supplement of dairy meal. The guinea pigs were on rat chow, while the

Abbreviations: GLC, gas–liquid chromatography (or) chromatographic; TLC, thin-layer chromatography; C_4 to C_{22}, fatty acids with 4 to 22 carbon atoms; C_{24} to C_{60}, triglycerides with a total number of fatty acid carbon atoms of 24 to 60; "16,16,18" and "4,18,18," etc., triglycerides made up of two C_{16} and one C_{18}, and of one C_4 and two C_{18} acids per molecule, etc.

dog was feeding on household refuse. The women were on free-choice diets.

Isolation of Triglycerides

The triglycerides were isolated from the globule fat which was prepared as follows. The milk (25 ml) was centrifuged for 30 min at 650 *g*. The serum was removed by siphoning with a Teflon capillary. The globules were suspended in 0.9% NaCl (25 ml) and recentrifuged. The saline was siphoned off and the washing repeated. After the final wash, the milk fat globules were extracted with 20 ml of chloroform–methanol 2:1 and the extract was washed once with saline (10 ml) and once with distilled water (10 ml). The phases were separated by centrifugation after each washing. The chloroform phase (15 ml) was dried over anhydrous magnesium sulfate (5 g) and filtered through a cotton plug inserted in the stem of a 25 ml glass funnel. The filtrate was evaporated at 37°C in a rotary evaporator and the residue taken up in chloroform.

TLC

Pure triglycerides of milk fat were obtained from the above extract by chromatography on thin layers (0.25 mm) of Silica Gel G. Approximately 25 mg of the total lipid was applied as a band along one edge of a 20 × 20 cm plate, and the plate was developed in heptane–isopropyl ether–acetic acid 60:40:4. The developed plates were allowed to dry in air for 1–3 min. The bands were located under UV light after the plate had been sprayed with a 0.05% solution of 2,7-dichlorofluorescein in 50% methanol. The band corresponding to triglycerides was marked and the silica gel of that area was immediately scraped off the plate and collected. The triglycerides were eluted from the gel with a solution (50 ml) of 5% methanol in diethyl ether by filtration through a cotton plug. The filtrate was evaporated to dryness under nitrogen and the residue suitably diluted for GLC.

GLC

A portion (10 mg) of the purified triglycerides was converted to butyl esters of the component fatty acids (7). A standard mixture of the butyl esters of C_4–C_{18} fatty acids was prepared by transbutylating an equal-weight mixture of simple standard triglycerides.

The butyl esters were analyzed in an F & M high efficiency gas chromatograph, model 402, equipped with dual glass columns, dual flame ionization detectors, and a differential electrometer. Two columns (100 cm × 0.25 cm I.D.) were packed with 15% diethylene glycol succinate polyester (DEGS) on Gas-Chrom P (60–80 mesh) and were conditioned at 250°C for 2 hr. The nitrogen flow rate was adjusted to 40 ml/min. Samples containing C_4–C_{18} acids were analyzed with temperature programming (70–220°C at 4°C/min rise), while samples of C_{10}–C_{18} acids were analyzed isothermally at 190°C. The detector and injector temperatures were maintained at 250°C. Quantitative results with fatty acid standards (National Heart Institute Mixtures E and F) agreed with the stated composition data with a relative error less than 2% for major components (over 10% of total mixture) and less than 5% for minor components (below 10% of total mixture).

GLC analyses of intact triglycerides were performed under the improved conditions described by Kuksis and Breckenridge (8). An Aerograph model 204B dual column instrument, equipped with dual hydrogen flame ionization detectors and a differential electrometer (Wilkens Instrument & Research, Inc., Walnut Creek, Calif.), was used in combination with an Electronik 15 Honeywell recorder and a disc integrator. 1 μl of a 1% solution of the triglyceride mixture was injected and the column temperature was programmed from 180 to 325°C at a rate of 3–6°C/min. Triglyceride recoveries were computed from runs with standard C_{24}–C_{54} glycerides. Values obtained from duplicate analyses showed a relative error of less than 3% for any peak comprising more than 5% of the sample, and less than 10% for any peak comprising less than 5% of the sample. Odd-carbon number triglycerides in the milk fat samples were estimated by completing the elution curves between the adjacent even carbon number triglycerides. These estimates showed a relative error of 10%.

RESULTS AND DISCUSSION

Work with standard mixtures of trioctanoin, trilaurin, trimyristin, tripalmitin, and tristearin showed that the peak areas recovered on GLC corresponded closely to the weight proportions of these glycerides in the injection solution. In relation to trioctanoin (C_{24}), which was assumed to be completely eluted, the other glycerides were recovered in the ratios of their original weights with an error of 1.1% or less. The percentage error in this case describes the maximum percentage deviation by which the estimated average (three to four chromatograms) proportion of the peak area differed from the corresponding weight proportion. This GLC system was therefore used, without correction factors, for the analysis of the unknown glycerides in the C_{24}–C_{54} range. The larger error, indicated under Materials and Methods for the individual triglyceride groups in the milk fats, was due to incomplete resolution of the peaks, and not to the incomplete recoveries of the glycerides from the column.

Figs. 1–3 are the GLC elution patterns recorded for the three main types of milk fat triglycerides. The pattern shown in Fig. 1 for guinea pig milk is similar to those obtained for the milk triglycerides of dog and man.

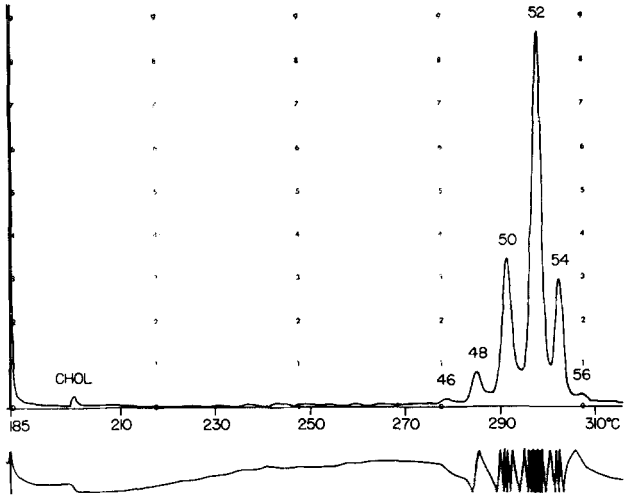

FIG. 1. GLC of triglycerides of guinea pig milk. Peaks are identified by the total number of carbon atoms in the fatty acid moieties. CHOL, cholesterol. Operating conditions as given in the figure and described in the text.

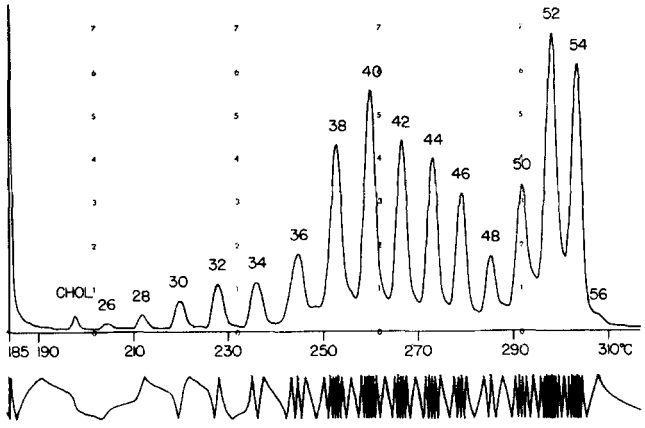

FIG. 2. GLC of triglycerides of goat milk.

In all of these fats the major glycerides were those with 48–54 acyl carbon atoms. Human milk was somewhat more complex than the other two, as it also contained measurable amounts of C_{40}–C_{46} and C_{56}–C_{60} glycerides. The relatively simple elution sequences of these glyceride mixtures are due to the virtual absence of short-chain fatty acids in these milk fats. The incomplete return of the recorder pen to the base line between adjacent peaks reflects the presence of odd-carbon number fatty acids, which give rise to small amounts of odd carbon number triglycerides.

Fig. 2 shows the triglyceride pattern of goat milk. It nearly matched that obtained for some samples of sheep milk. The glyceride patterns of both sheep and goat milk resembled closely those of the cow's milk. However, the sheep and the goat have proportionally more of the C_{54} component than the cow, and there are differences

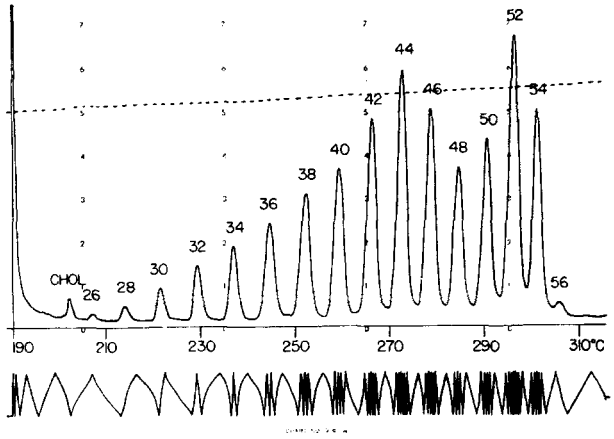

FIG. 3. GLC of triglycerides of horse milk.

among these three species in the location of the first maximum, as well as the minimum, of the glyceride population. Differences were also noted between the triglyceride patterns of the milk fats of the Jersey and Holstein breeds of cows. The average molecular weight of the Jersey milk triglycerides was lower than that of the triglycerides of the Holstein milk. This was due to a larger proportion of the short-chain fatty acids in the Jersey milk. Samples of Jersey milk analyzed by others (9), however, have shown higher proportions of the longer-chain fatty acids than those reported in Table 1. Hence the differences between the milks of the Holstein and Jersey breeds noted in this study may not be typical.

The elution pattern shown for the horse milk triglycerides in Fig. 3 is characterized by a nearly complete return of the recorder pen to the base line between adjacent peaks, which indicates a relative absence of odd-carbon number triglycerides from this fat. In common with goat milk, the distribution has a minimum at C_{48}, but the first maximum is displaced towards the longer chain lengths (C_{44}). The fats of all the milks had the same "second maximum" at C_{52}.

Table 1 gives the fatty acid composition of the purified milk fat triglycerides. The use of butyl esters in the GLC analysis prevented losses of short-chain acids by volatilization and provided for all fatty acids a nearly uniform ion yield in the hydrogen flame ionization detector. The present data show qualitative and quantitative differences from comparable previously reported analyses. The divergence is greatest for the milk fat of horse, which had been examined earlier by ester distillation (10). For the acids of the milk triglycerides of cow (3) and man (2), where GLC analysis had been used, the agreement with the present values is good, differences being attributable to subject variation. The values for the fatty acids of goat and sheep milk, which had previously been determined (11) by GLC of the methyl esters, in

TABLE 1 FATTY ACID COMPOSITION OF MILK FAT TRIGLYCERIDES

Fatty Acids*	Dog	Guinea Pig	Man	Cow: Jersey	Cow: Holstein	Goat	Sheep	Horse
				moles %				
4:0	—	—	—	9.8	8.5	8.2	10.3	—
6:0	—	—	—	5.0	2.9	6.9	3.4	0.7
8:0	—	—	—	2.4	1.4	5.8	2.3	5.4
10:0	tr.	—	0.6	4.8	2.3	7.9	3.4	12.3
12:0	0.4	tr.	3.0	4.1	2.1	1.9	1.8	8.5
14:0	3.6	3.1	5.3	11.8	7.5	2.6	5.0	6.9
15:0	1.4	0.7	0.6	1.7	1.2	0.7	0.9	0.2
16:0	23.6	30.9	26.5	36.5	28.0	16.0	20.9	21.3
16:1	5.1	3.3	4.0	1.1	1.6	1.2	1.2	4.5
16:2	1.4	—	—	—	—	—	—	—
17:0	2.6	1.0	1.1	0.8	0.7	2.4	2.9	0.5
18:0	8.5	3.0	7.8	8.6	14.6	14.3	15.5	2.1
18:1	41.5	39.0	37.6	13.0	26.5	30.4	27.2	17.4
18:2	8.9	17.1	10.0	0.4	1.5	1.7	2.9	14.7
18:3	—	—	0.6	—	1.0	—	2.4	5.6
20:0	2.4	2.0	—	—	tr.	—	tr.	—
20:1	0.6	—	0.6	—	—	—	—	—
20:2	—	—	0.5	—	—	—	—	—
20:3	—	—	0.4	—	—	—	—	—
20:4	—	—	0.8	—	—	—	—	—
22:2	—	—	0.3	—	—	—	—	—
22:5	—	—	0.1	—	—	—	—	—
22:6	—	—	0.3	—	—	—	—	—

* Shorthand designation adopted from Farquhar, Insull, Rosen, Stoffel, and Ahrens (12).

some cases differed markedly from those reported herein. No reference could be located to comparable previous analyses of the fatty acids of the milk triglycerides of guinea pig and dog.

Table 1 shows that the milk triglycerides of these species have characteristic fatty acid compositions. Those of the dog, guinea pig, and man contain no fatty acids of a chain length shorter than 10 carbon atoms. The milk fats of the ruminants contain up to 20 mole % of the total fatty acid in the form of C_4–C_8 compounds. Horse milk contains no butyric acid and little caproic acid, but is rich in capric acid (12.3 mole %). The milk triglycerides of man are characterized by the presence of significant proportions of C_{20} and C_{22} unsaturated fatty acids, incorporated into triglycerides containing 56–58 acyl carbon atoms.

The proportion of the odd-carbon number fatty acids is lowest in the horse milk (0.61 mole %) and highest in the dog milk (4 mole %). These acids also make up about 3 mole % of the sheep milk fatty acids.

Some of the differences between the milk fat acids of the different species are of the order of those noted for the acids of the milk fats of the two breeds. Thus, while both the Jersey and Holstein cows produced milks of about the same butyric acid content, the Jersey milk contained considerably more of the other short-chain fatty acids, as well as more myristic and palmitic acids than did the Holstein milk, which in turn was richer in stearic and oleic acids.

Tables 2 and 3 give the quantitative molecular weight distributions of the milk fat triglycerides in the seven species investigated. Table 2 compares the experimental values obtained for dog, guinea pig, and man with the expected values as calculated from random distribution. Despite the relatively small number of the triglyceride types, the deviations from random distribution are obvious and significant. An inspection of the table shows that in many cases the differences between the calculated and experimental estimates exceed the relative error of the measurement five-fold or more. The difference is least for the dog milk, but even there considerable variation can be seen in the relative contributions of the C_{48} and C_{54} components. The calculations consistently underestimate the shorter- and overestimate the longer-chain triglycerides. The greatest differences between expected and actual values occurred in the distribution of the triglycerides of the guinea pig milk. In it the random values for the C_{52} component is very much lower (36.7%) than the experimental value (53.1%), and the random value for the C_{54} component (23.1%) is very much higher than its experimental value (13.8%). This suggests that in the guinea pig there is marked preferential synthesis of the 16, 18, 18 triglycerides in relation to that for the 16, 16, 18 and the 18, 18, 18 triglycerides. A preferential formation of 16, 18, 18 triglycerides may also be noted for the human milk in which the experimental value (39.0%) greatly exceeds the random value (29.5%).

TABLE 2 TRIGLYCERIDE COMPOSITION OF THE MILK FATS OF DOG, GUINEA PIG, AND MAN

Triglycerides*	Dog: Experimental	Dog: Random†	Guinea Pig: Experimental	Guinea Pig: Random	Man: Experimental	Man: Random
			moles %			
38	—	—	—	—	tr.	tr.
40	—	—	—	—	0.4	0.2
42	—	—	—	—	1.3	0.7
44	—	—	—	—	2.8	2.1
45	—	—	—	—	tr.	0.2
46	4.9	1.7	0.9	1.3	5.5	5.6
47	2.2	0.7	tr.	0.3	tr.	0.6
48	10.3	7.0	5.0	7.8	9.0	11.7
49	2.6	2.5	0.9	1.0	0.8	1.0
50	21.6	20.9	21.1	24.1	17.6	21.2
51	3.2	4.3	2.5	1.9	1.2	1.6
52	32.5	32.5	53.1	36.7	39.0	29.5
53	2.1	3.0	1.5	1.0	0.8	1.0
54	17.4	23.7	13.8	23.1	16.4	20.3
55	tr.	0.3	—	—	tr.	0.1
56	3.5	3.1	1.2	2.2	3.6	3.0
58	tr.	0.2	—	—	1.4	0.2
60	—	—	—	—	0.3	0.1

* Triglycerides are identified by the total number of acyl carbon atoms per glyceride molecule.

† Calculated using probability equations (4).

TABLE 3 TRIGLYCERIDE COMPOSITION OF THE MILK FATS OF COW, GOAT, SHEEP, AND HORSE

Triglycerides*	Jersey		Holstein		Goat		Sheep		Horse	
	Experimental	Random†	Experimental	Random	Experimental	Random	Experimental	Random	Experimental	Random
	moles %									
12–24	0.0	3.1	0.0	1.2	0.0	2.6	0.0	1.9	0.0	0.0
26	0.1	2.2	tr.	1.6	0.3	2.0	0.3	2.2	0.3	0.2
28	0.5	2.0	0.5	1.1	0.8	2.5	0.5	1.4	0.8	0.4
30	1.0	2.3	0.7	1.0	1.7	3.3	1.0	1.6	1.5	1.0
31	0.1	0.1	—	—	0.2	0.2	—	—	—	—
32	2.2	2.9	1.3	1.4	2.6	3.2	1.6	1.8	2.9	1.2
33	0.4	0.2	—	0.1	0.3	0.1	—	0.1	—	—
34	7.3	4.5	3.5	2.1	2.9	2.0	2.8	1.9	3.9	2.2
35	1.6	0.5	0.6	0.3	0.3	0.2	1.0	0.2	—	—
36	14.4	8.2	9.4	4.7	5.3	3.7	6.3	3.8	5.9	5.0
37	1.2	0.7	1.0	0.3	0.8	0.6	1.3	0.7	—	—
38	13.7	9.4	15.9	9.8	10.7	7.0	13.0	8.1	7.3	5.6
39	0.7	0.5	0.8	0.3	1.3	0.8	1.7	1.6	—	—
40	9.3	7.0	11.6	8.3	12.8	11.0	12.1	10.4	8.4	6.8
41	0.6	0.5	0.4	0.2	0.8	0.6	0.8	0.5	—	0.1
42	7.0	5.9	5.7	4.2	9.3	8.6	5.5	5.0	10.4	9.8
43	0.2	0.5	0.3	0.2	0.7	0.6	0.3	0.4	—	0.2
44	6.7	6.7	3.6	4.1	7.8	7.9	4.5	4.5	12.4	13.5
45	0.7	0.7	0.2	0.3	0.7	0.9	0.6	0.5	—	0.3
46	7.2	8.6	3.8	5.7	5.8	6.4	4.1	4.6	10.2	13.0
47	1.0	1.2	0.4	0.5	0.3	0.3	0.6	0.5	—	0.2
48	8.2	11.8	5.6	9.7	2.7	3.0	5.0	5.5	7.0	10.0
49	1.0	0.5	0.7	1.3	0.6	0.7	1.0	1.3	—	0.3
50	8.2	11.1	10.9	15.8	6.4	5.8	9.6	10.5	8.3	11.1
51	0.5	0.7	0.9	1.2	1.0	1.6	1.0	2.3	—	0.4
52	5.1	5.5	14.7	16.9	12.7	11.1	13.5	15.3	12.3	12.2
53	0.2	0.1	0.5	0.4	0.8	1.5	1.2	2.0	—	0.2
54	1.0	1.1	7.0	8.3	10.5	10.0	10.5	11.1	8.0	6.3
56	—	—	0.2	tr.	tr.	—	0.5	—	0.6	—

* Triglycerides are identified by the total number of acyl carbon atoms per glyceride molecule.
† Calculated using probability equations (4).

Table 3 compares the experimental and random distributions for the glycerides of ruminant and horse milks. Again the deviations from chance distribution are obvious. In all cases the calculations overestimate the longer-chain triglycerides (C_{46}–C_{54}) and underestimate the shorter-chain triglycerides (C_{34}–C_{42}). In this respect the data for the bovine milks compare favorably to those for butterfat triglycerides prepared from pooled milk (5). The peculiar triglyceride distribution of the equine milk is largely due to the unusually high content of capric acid in this milk. Again a nonrandom pattern is apparent for most glyceride types. This difference in the distributions cannot be explained by an incomplete recovery of the long-chain triglycerides from the GLC column, because calculations of the recovery of the fatty acid carbons (5) as well as reconstitution studies with several samples of bovine milk have shown better than 96% recovery of all components. The differences in the glyceride distributions are therefore real and can be best understood on the basis of the proposal that contributions to milk fat synthesis vary according to the lipid supplies from several separate pools (2, 10).

The amount of the long-chain glycerides in the present goat milk sample (Table 3) is much higher than that indicated by the GLC separations of Dimick and Patton (6). Differences in the fatty acid composition of the samples, and in the distribution of the triglycerides and (or) incomplete recoveries from the GLC column could account for this.

At this level of experimental examination all of the ruminant milks appear to possess similar structures with only one of the short-chain fatty acids appearing in any one glyceride molecule. The C_{34}–C_{40} glycerides which are present in amounts exceeding the random values, must all contain (though not exclusively) one short-chain acid in combination with two C_{14}–C_{18} fatty acids per molecule. This conclusion is supported by the isolation of high proportions of triglycerides of types 4, 16, 16 and 4, 16, 18 from molecular distillates of butter oil (7). It would therefore be anticipated that the horse milk would contain significant amounts of the triglycerides of types 16, 16, 10 and 16, 18, 10, as is borne out by the demonstration of major peaks for the C_{42} and C_{44} triglycerides in its GLC elution pattern.

Further insight into the composition of these milk fats can be obtained by GLC following a preliminary segregation of these glycerides on thin-layer plates impregnated with silver nitrate (7). Such studies are in progress in this laboratory. The present data serve to substantiate the claims (4) that the synthesis of milk triglycerides is a nonrandom process.

These studies were supported by grants from the Ontario Heart Foundation, Toronto, Ontario, and the Medical Research Council of Canada.

Manuscript received 20 February 1967; accepted 2 June 1967.

References

1. James, A. T., and A. J. P. Martin. 1956. *Biochem. J.* **63:** 144.
2. Insull, W., Jr., and E. H. Ahrens, Jr. 1959. *Biochem. J.* **72:** 27.
3. Herb, S. F., P. Magidman, F. E. Luddy, and R. W. Riemenschneider. 1962. *J. Am. Oil Chemists' Soc.* **39:** 142.
4. Jensen, R. G., and J. Sampugna. 1966. *J. Dairy Sci.* **49:** 460.
5. Kuksis, A., M. J. McCarthy, and J. M. R. Beveridge. 1963. *J. Am. Oil Chemists' Soc.* **40:** 530.
6. Dimick, P. S., and S. Patton. 1965. *J. Dairy Sci.* **48:** 444.
7. Kuksis, A., and W. C. Breckenridge. 1965. *J. Am. Oil Chemists' Soc.* **42:** 978.
8. Kuksis, A., and W. C. Breckenridge. 1966. *J. Lipid Res.* **7:** 576.
9. Stull, J. W., and W. H. Brown. 1964. *J. Dairy Sci.* **47:** 1412.
10. Garton, G. A. 1963. *J. Lipid Res.* **4:** 237.
11. Freeman, C. P., E. L. Jack, and L. M. Smith. 1965. *J. Dairy Sci.* **48:** 853.
12. Farquhar, J. W., W. Insull, Jr., P. Rosen, W. Stoffel, and E. H. Ahrens, Jr. 1959. *Nutrition Revs.* **17** (8, Pt. 2): August Supplement.