The Incorporation of ^{14}C-Glycerol into Different Species of Diglycerides and Triglycerides in Rat Liver Slices

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ABSTRACT

The relative rates of de novo synthesis of species of diglycerides and triglycerides from ^{14}C-glycerol were examined in rat liver slices. Diglycerides containing one or two double bonds per molecule and triglycerides containing four or more double bonds per molecule represented 70% and 60% respectively of the newly synthesized diglycerides and triglycerides. The newly synthesized triglycerides were more unsaturated than the endogenous triglycerides. Our results suggest that a nonrandom synthesis of species of diglycerides occurred followed by an almost random utilization of the various diglyceride species for the biosynthesis of triglycerides.

INTRODUCTION

In liver 1,2-diglycerides can be considered to serve as a common precursor for the biosynthesis of glyceryl esters triglycerides, lecithin and cephalin. Since the formation of all three types of lipid requires reaction at only the 3 position, the ordered structure of the diglycerides would be expected to remain as a common feature of the product glyceryl esters. Saturated and unsaturated fatty acids are predominantly present at the 1 and 2 positions respectively of triglycerides, lecithin and cephalin (1-3), but differences in fatty acid composition of these types of lipids have been observed. For example, stearic and arachidonic acids, which are major components of rat liver lecithin and cephalin, are minor components in rat liver triglycerides (1, 4). Such structural studies indicate that the distribution of fatty acids in glyceryl esters is not a random process, but rather that mechanisms exist in the cell which control both the positional occurrence and the extent of occurrence of a given fatty acid in different glyceryl esters.

Attempts to demonstrate the reactions which control the location of a given fatty acid have not been overly successful. Experiments testing the specificity for certain diglycerides in the enzymic acylation of diglycerides to produce triglycerides have been reported (5, 6); however, the significance of these studies was obscured by differences in solubility of different diglycerides. Likewise, no appreciable specificity has been observed in the formation of phosphatidic acid by acylation of glycerol-3-phosphate (7) or in the hydrolysis of phosphate from phosphatidic acid (8).

In the present study the de novo synthesis of diglycerides and triglycerides from ^{14}C-glycerol was examined to see if all types of triglyceride were formed at similar rates. Our results indicate that a degree of selectivity occurred in the synthesis of triglycerides by rat liver, and that the de novo synthesis of triglyceride did not lead to a duplication of the pre-existing distribution of molecular species of triglycerides.

EXPERIMENTAL

Incorporation of ^{14}C-Glycerol into Liver Lipids

Male Sprague-Dawley rats (175-240 g) were sacrificed by decapitation. The body cavity was immediately opened, and the liver was perfused with cold Locke's solution (9) devoid of calcium containing 0.027 M trisodium citrate until the liver attained a creamy color. Liver slices were prepared with a Briggs-Stadie microtome.

Liver slices (2 to 3) were incubated in Krebs-Ringer bicarbonate buffer (9) containing one-half the normal amount of calcium with 100 μmoles of ATP, 0.2 mg of reduced coenzyme A and 10 μc of 1-^{14}C-glycerol (10 μc/μm). The final volume of the incubation mixture was 7.5 ml. Incubations were performed at 37C with constant shaking in an atmosphere of 5% carbon dioxide and 95% oxygen. Liver slices were removed at indicated time intervals, rinsed, and immediately frozen between 2 blocks of dry ice.

Extraction of Lipids

After the weight (200-400 mg) of the frozen tissue had been recorded, the sample was homogenized in 5 ml of chloroform-methanol (1:1, v/v) containing 100 μl of a solution of 1% 2,6-di(t-butyl) 4-methylphenol in chlo-

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roform with a Virtis microhomogenizer for 1 min. An additional 2.5 ml of chloroform was added, and the homogenization was continued for approximately 30 seconds. Additional chloroform-methanol (2:1) was added to give a final volume of 20 ml, and the samples were allowed to remain at room temperature for approximately 30 min. The extracts were washed once with 0.2 volumes of 0.58% sodium chloride and once with "pure upper phase" as described by Folch et al. (10). The samples were evaporated to dryness using a rotary evaporator which was flushed with nitrogen, dissolved in chloroform and stored at –15C.

Isolation of Neutral Lipids

Neutral lipids were separated from polar lipids using DEAE-cellulose columns as described by Rouser et al. (11). Diglycerides and triglycerides were isolated by thin-layer chromatography (TLC) using Mallinckrodt silicic acid of less than 200 mesh. The plates were developed 4 cm in diethyl ether-petroleum ether (4:1). After drying for 15 min at room temperature, they were developed in diethyl ether-petroleum ether (12:88) to a distance of 18-20 cm. The lipids were visualized under ultraviolet light after spraying the plate with a solution of 0.2% 2′,7′-dichlorofluorescein in methanol. The bands of triglycerides (R_f = 0.70) and diglycerides (R_f = 0.30) were scraped from the plate and the lipids were eluted with methanol-ether (1:9).

An aliquot of the recovered triglyceride fraction revealed a radiochemical purity of less than 90% when chromatogrammed with carrier diglycerides and triglycerides. Accordingly, the whole triglyceride samples were routinely rechromatographed on thin layer plates as described above; to give a radiochemical purity of greater than 95%.

Similarly, the isolated diglycerides were found to be contaminated with small amounts of cholesterol and an unidentified impurity. Since these contaminants yielded anomalous bands in the subsequent separation of the diglyceride fraction into species, the diglycerides were further purified by TLC in a solvent of chloroform-acetone (96:4) with a layer of boric acid-Adsorbosil-1 (12). Approximately 90% of it was located in the 1,2-diglyceride fraction and more than 95% of the isotope was recovered. The remainder of the radioactivity was found in nearly equal amounts in monoglyceride, 1,3-diglyceride and triglyceride fractions.

Separation of Di- and Triglycerides into Molecular Species

The separation of diglycerides into individual species was achieved using thin layer plates containing 5% (w/w) silver nitrate Adsorbosil-1. Thin layers (0.3 mm) were activated at 125C for 75 min and stored over a saturated solution of calcium chloride in a metal cabinet.

Diglycerides prepared from pig liver lecithin by the action of phospholipase C (13) were added as a carrier to all diglyceride samples to facilitate location of the samples in subsequent steps. The plates were developed in a solvent system composed of diethyl ether-petroleum ether-benzene-methanol (45:35:30:2). After spraying the plates lightly with a solution of 0.1% of 2′,7′-dichlorofluorescein in methanol five discrete bands were visible under ultraviolet light. These bands were found to contain one to five or more double bonds per

TABLE I
Fatty Acid Composition of the Carrier Diglyceride Resolved by Silver Nitrate-TLC

Band[a]	16:0	18:0	18:1	18:2	18:3	20:3	20:4	22:4	22:6	Band designation
	Fatty acid, Mole %									
4	20	29	45	5	2	—	—	—	—	
[b]	19	30	44	6	2	—	—	—	—	Monoene
5	13	35	4	49	—	—	—	—	—	
[b]	14	33	7	46	—	—	—	—	—	Diene
7	10	44	4	—	18	25	—	—	—	
[b]	10	42	3	10	17	28	—	—	—	Triene
8	12	43	4	—	1	1	35	5	—	
[b]	13	48	4	—	—	—	36	—	—	Tetraene
9	17	41	3	—	1	1	1	—	28	
[b]	17	41	3	—	1	4	—	—	34	Polyunsaturated
Total	16	35	20	13	3	4	4	1	4	

[a] The TLC-plate was divided into ten bands. Less than 3% of the total fatty acids was located in bands other than those indicated in this table.

[b] The second row represents a separate experiment.

molecule by means of GLC analysis of the methyl esters derived from the diglycerides. The *fatty acid composition* of these bands is indicated in Table I. Less than 3% of the total diglyceride sample applied to the TLC plate was recovered in areas other than the five bands described in Table I.

The method used to separate the triglyceride fraction of rat liver into its component species has been described in a previous communication from this laboratory (14).

Elution of Di- and Triglycerides from Silver Nitrate-Silicic Acid

The bands of silicic acid that contained the separated glycerides were scraped from the thin layer plates and transferred into small test tubes. A solution of 1% sodium chloride in 90% methanol was added in portions (approximately 0.5 ml) with vigorous mixing until the characteristic red color of the silver-dichlorofluorescein complex was destroyed. The glycerides were extracted with 5 ml of ether-methanol (9:1). After centrifugation, the supernatant solution was transferred to a scintillation vial. The residue was washed two additional times. The combined extracts were evaporated to dryness under a stream of nitrogen with gentle heating.

The triglycerides were counted in 7.0 ml of a scintillation fluid containing 4 g of 2,5-diphenyloxazole and 50 mg of p-bis[5-phenyloxazoyl]-benzene per liter of toluene. Diglycerides were counted in 10.0 ml of scintillation fluid prepared with dioxane (15). The recovery of radioactivity applied to the plate was greater than 90% in either case.

Gas-Liquid Chromatography

These methods have been described in an earlier paper (14).

MATERIALS

All solvents were analytical reagent grade and were used without further purification. The petroleum ether had a boiling point range of 30-60C. DEAE-cellulose (DE-23) was obtained from Reeve Angel Corp.

RESULTS

The time course of incorporation of ^{14}C-glycerol into diglycerides and triglycerides is shown in Fig. 1. The total counts incorporated into neutral lipids increased in a linear fashion for 2-3 hr. Preliminary results showed that optimal yields of lipid soluble ^{14}C-glycerol were obtained when ATP and CoA were added to the incubation medium. In addition, the lower Ca^{++} content led to more radioactivity in the neutral lipids. The total counts present in the diglyceride and triglyceride fractions were nearly equal at each time interval measured. Since the quantity of triglyceride in rat liver is much greater than that of diglyceride, the specific activity of the diglyceride pool at a given time interval was much greater than that of the triglyceride pool.

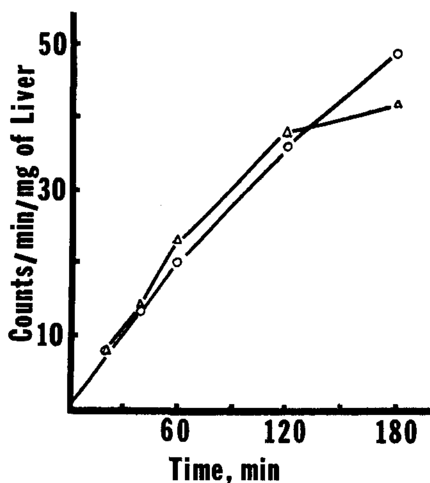


FIG. 1. The incorporation of ^{14}C-glycerol into diglycerides and triglycerides in rat liver slices. ○——○ Diglycerides, △——△ Triglycerides.

The percentage of distribution of ^{14}C-glycerol among the species of triglycerides at various time intervals is shown in Table II. After 20 min of incubation approximately 70% of the incorporated label was located in three fractions, the SMD, SD_2 and the polyunsaturated species. The other six species indicated contained less than 30% of the incorporated label. The distribution of incorporated glycerol among the species had been established at the first time point measured. This pattern was not altered upon extended incubation even though the total incorporation of ^{14}C-glycerol increased several fold.

The mole percent distribution of triglyceride species of unincubated rat liver and two incubated samples is shown in Table III. The SM_2, SMD and polyunsaturated subfractions of triglycerides were the major components to accumulate ^{14}C-glycerol, and they comprised 16, 24 and 22% respectively of the total mass represented by the triglyceride fraction. The *weight distribution* of species of triglycerides did not demonstrably change during the course of the incubation.

Comparison of the distribution of ^{14}C-glycerol among species to the weight distribution of species shows that the de novo synthesis of

TABLE II
Incorporation of ^{14}C-glycerol into Species of Triglycerides

	Percentage Distribution of Counts					
	20 min	40 min	60 min	90 min	120 min	180 min
Subfractions[a]	%					
S_3	1	0	1	1	0	1
S_2M	3	2	1	2	3	3
SM_2	8	5	6	6	6	5
$S_2D + M_3$	8	7	10	8	9	8
SMD	16	15	16	15	16	14
M_2D	7	5	5	5	4	4
SD_2	11	13	15	14	14	12
MD_2	6	7	5	6	5	5
Polyunsaturated[b]	42	46	39	44	44	47

[a] S,M and D are used as abbreviations for saturated, monounsaturated, and diunsaturated fatty acids respectively. The subscript refers to the number of times a given acid appears in the same species of triglyceride.

[b] Polyunsaturated triglycerides represent several slow moving bands which were combined. These triglycerides contain at least one fatty acid containing five or more double bonds or they contain six or more double bonds by virtue of the presence of more than one unsaturated fatty acid.

TABLE III
Distribution of Individual Species of Triglycerides in Rat Liver

Species	Unincubated[a]	20 min[b]	120 min[b]
	Mole %		
S_3	1	1	2
S_2M	6	4	8
SM_2	14	16	20
$S_2D + M_3$	8	8	8
SMD	26	24	23
M_2D	8	7	4
SD_2	11	10	9
MD_2	5	6	5
Polyunsaturated	22	23	21

[a] The value represents the average of three different triglyceride samples isolated from whole liver. The values are corrected for cross contamination.

[b] A portion of the triglyceride sample from liver slices incubated with ^{14}C-glycerol was separated into species by silver nitrate-TLC. The individual species of triglycerides were converted to methyl esters for analysis by GLC.

triglycerides from glycerol did not produce new molecular species in proportion to their endogenous abundance. Certain species, SM_2 and SMD, incorporated approximately one-half the amount of isotope that would be predicted from their original abundance. Conversely, the polyunsaturated species incorporated approximately twice the amount of isotope that would be predicted from the weight of this subfraction.

In Table IV the distribution of ^{14}C-glycerol among the species of diglycerides is shown. Shorter periods of incubation were examined in this experiment since no alterations in labeling pattern were observed with species of triglycerides from incubations of 20 min or longer. As indicated earlier, the observable bands were due to the molecular species of added diglyceride that had been prepared from pig liver lecithin; however, no appreciable amount of radioactivity was detected in the other areas in which bands were not discernible. In the present experiments, the diglycerides were separated according to the total number of double bonds per molecule. Thus a diglyceride containing 2 monoenoic acids and a diglyceride containing a saturated acid and a dienoic acid were not separated from one another. In contrast to the results with triglycerides, the distribution of ^{14}C-glycerol among the species of diglycerides was predominantly in the more saturated species rather than in the unsaturated species. At all time points examined, approximately 70% of the isotope was found in the monoenoic and dienoic diglycerides. With increasing times of incubation, the isotopic content of the tetraene fraction gradually increased. This percentage increase was compensated by a decrease in the relative radioactivity of the diene fraction. Other species contained a more or less constant percentage of the incorporated ^{14}C-glycerol throughout the course of the incubation.

When the ^{14}C-diglycerides were subjected to methanolysis with sodium methoxide, 2-4% of the recovered counts appeared in the methyl esters regardless of the length of the incubation period. The recovery of counts in methyl esters plus glycerol ranged from 90-93%. Thus,

TABLE IV
The Incorporation of ^{14}C-glycerol into Species of Diglycerides

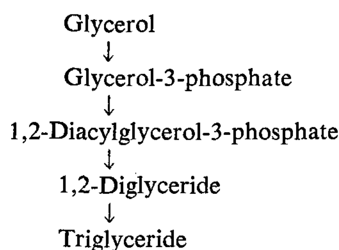
Number of double bonds	Percentage Distribution of Counts									
	5 min[a]	10 min[a]	15 min[a]	20 min[a]	30 min	40 min[a]	60 min[a]	90 min[a]	120 min	180 min
	%									
1	24	26	24	24	25	23	28	28	27	29
2	51	48	48	46	43	41	40	40	40	42
3	5	5	5	5	5	5	4	4	4	4
4	6	7	8	10	11	14	14	13	12	11
5	10	12	10	10	9	14	10	10	13	11
Origin	3	2	5	5	7	4	4	6	2	3

[a] These values are the average of two separate experiments.

the labeling patterns observed are representative of the incorporation of ^{14}C-glycerol into the glycerol portion rather than the fatty acid of the diglyceride.

DISCUSSION

The present experiments were designed to consider two questions. First, does the biosynthesis of triglycerides from diglycerides involve a random acylation of diglyceride units from a pool of diglycerides or does the synthesis preferentially select certain diglyceride units? Secondly, are fatty acids randomly incorporated into the newly-synthesized species of diglycerides? The pathway by which ^{14}C-glycerol can be incorporated into these pools of lipids is summarized below.



The de novo synthesis of triglycerides from glycerol did not yield the same relative amounts of species which are endogenous to the entire liver. The differences that we observed in the distribution of mass and radioactivity can be attributed to the presence of a separate pool which is rapidly synthesized and does not represent the triglycerides of the whole liver. Stein and Shapiro (16) suggested the presence of two pools of triglyceride, one of which rapidly equilibrates with plasma triglyceride and a second pool which is relatively inert. Other workers (17, 18) have also found that those triglycerides synthesized for secretion into plasma represent a more active pool.

Approximately 70% of the ^{14}C-glycerol incorporated into diglycerides was located in the monoene and diene fractions. On the other hand, approximately 60% of the total radioactivity in triglycerides is present in those species of triglycerides containing four or more double bonds per molecule. Since acylation of the 3 position can introduce further unsaturation into the molecule, it is not surprising that the degree of unsaturation of the product triglyceride is greater than that of the precursor-diglycerides. The unsaturated character of the 3 position of rat liver triglycerides has been emphasized in a recent report from this laboratory (14).

To compare the degree of unsaturation of the newly-formed triglycerides to their precursor diglycerides, we asssumed that the fatty acid composition at the 3 position might be similar to that reported. If the diglyceride units were randomly esterified in a non-correlative manner, we could predict the expected quantities of isotope in the triglyceride species by multiplication of the mole fractional occurrence of a given fatty acid at the 3 position of triglyceride times the percentage distribution of isotope in the newly-synthesized diglycerides. For example, diglyceride species containing 2 double bonds per molecule constituted 46% of the total radioactive diglycerides. This species of diglyceride will lead to the formation of SDX or MMX triglycerides (X denotes the fatty acid esterified to the 3-position). If X is saturated, the triglyceride will contain only two double bonds whereas if X is a monene the triglyceride will contain three double bonds, etc. For simplicity, we considered only the total number of double bonds per molecule. The extent to which each type of diglyceride would be expected to produce the different triglyceride species is shown in Table V.

TABLE V
Predicted Distribution[a] of Species in Newly-Synthesized Triglycerides

Precursor[b] diglyceride	Expected number of double bonds in triglyceride						
	0	1	2	3 %	4	5	≥6
Monoene	—	3	9	5	1	1	6
Diene	—	—	6	18	9	1	12
Triene	—	—	—	1	2	1	1
Tetraene	—	—	—	—	1	4	5
Polyunsaturated	—	—	—	—	—	—	15
Predicted	0	3	15	24	13	7	39
Observed[c]	1	3	16	16	18	6	42

[a]The expected (predicted) percentage of isotope in species of triglycerides is estimated by multiplication of the percentage of isotope in a given species of diglyceride times the fractional occurrence of fatty acids at the 3 position of endogenous triglycerides. The composition of the 3 position is 12, 39, 20, 3, 3 and 23% respectively for saturated, monoenoic, dienoic, trienoic, tetraenoic and unidentified acids (14).

[b]These values are taken from Table IV, 20 min.

[c]These values are taken from Table II, 20 min.

For those triglycerides containing 1, 2, 5 and 6 or more double bonds per molecule, the predicted values for radioactivity agree very closely with the observed values. These calculations, however, predict somewhat higher and lower values respectively for those triglycerides containing four and five double bonds per molecule. Similar calculations for other periods of incubations yielded analogous results. The close correlation between the predicted and observed values leads us to believe that very little selectivity occurred in the

utilization of diglyceride species for triglyceride biosynthesis, and that acylation of the diglycerides proceeded with non-correlative specificity as had been previously suggested (14).

The observed distribution of ^{14}C-glycerol among diglyceride units indicates that the newly-synthesized diglycerides were composed of about 25, 45, 5 and 10% respectively of monoenoic, dienoic, trienoic and tetraenoic diglycerides. The total amount of ^{14}C-glycerol in all diglyceride fractions increased severalfold with time. The only significant variations observed in the percentage distribution of isotope among the diglyceride units were a twofold increase in the tetraenoic fraction and a slight decrease in the relative percent of label found in the diene fraction. The increase in radioactivity of the polyunsaturated diglycerides could arise in part from the hydrolysis at the 1 position of a radioactive triglyceride containing a polyunsaturated fatty acid at the 3 position. This reaction would be expected to contribute greater amounts of isotope to diglycerides with longer times of incubation, and would eventually tend to produce triglycerides with similar acid composition at the 1 and 3 positions. The small percentage of saturated and trienoic diglycerides compared to the large percentage of monoenoic and dienoic diglycerides indicates that the acylation of glycero-3-phosphate was not a random process, but rather that certain diglyceride units were formed in preference to others.

The diglyceride pool in livers of young rats that had been fasted and refed carbohydrate was reported to contain approximately 40, 30 and 20% respectively of monoene, diene plus triene and tetraene diglycerides (19). The content of monoenoic acids was presumably higher than normal due to the increased stearoyl-CoA desaturase activity under these conditions (20, 21). In view of the known increase in monoene diglyceride units and decrease in polyene diglyceride units of glycerolipids evoked by fasting and refeeding (13), our results on the newly synthesized diglycerides would seem to reflect the distribution of mass among diglycerides.

Lecithins may also form diglycerides by the reverse action of CDP-choline:diglyceride choline phosphotransferase, although this reaction does not represent net synthesis of diglyceride. Bjørnstad and Bremer (22) demonstrated a rapid reversibility of the CDP-choline:diglyceride choline phosphotransferase reaction in vivo in rat liver; however, it is not known whether all species of lecithin take part equally in this reversible reaction. Elovson showed that ^{14}C-stearic acid appeared in rat liver phospholipids at a much faster rate than would be predicted from the amount of isotope appearing in the diglycerides, whereas oleic acid derived from stearic acid appeared more rapidly in diglycerides than in phospholipids (19). He suggested that the stearic acid was preferentially incorporated into liver phospholipids via acylation of monoacyl phospholipids rather than by the action of the aminophosphotransferase on the diglyceride unit.

Nevertheless, transfer of ^{14}C-glycerol from phospholipids to triglycerides via diglyceride was expected to be minimal at early time points in these experiments since the labeled phospholipid would be greatly diluted by the large size of the endogenous pool. Thus, the contribution of radioactivity to the diglyceride pool by units derived from choline and ethanolamine phospholipid should be very small compared to that of ^{14}C-diglyceride arising by de novo synthesis.

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