Acyl specificity in triglyceride synthesis by lactating rat mammary gland

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Abstract We have studied the specificity of the acyl-CoA: diglyceride acyltransferase reaction in lactating rat mammary gland to provide a rational explanation at the enzyme level for the nonrandom distribution of fatty acids in milk fat triglycerides. Acyl-CoA:diglyceride acyltransferase activity was measured using various diglyceride and radioactive acyl-CoA substrates; products were identified as triglycerides by thin-layer and gas–liquid chromatography. Most of the enzymatic activity was located in the microsomal fraction and showed a broad specificity for the acyl donors tested (C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, and $C_{18:1}$ CoA esters). The acyltransferase activity was highly specific for *sn*-1,2-diglyceride enantiomers; *rac*-1,3- and *sn*-2,3-diglycerides were relatively inactive. The acyl-CoA specificity was not affected by the type of 1,2-diglyceride acceptor offered, although dilaurin was the best acceptor and *sn*-1,2-dilaurin > *sn*-1,2-dimyristin > *sn*-1,2-dipalmitin > *sn*-1,2-distearin. We have previously shown that in the microsomal fraction from lactating rat mammary gland, the acyltransferase activities concerned with the conversion of *sn*-glycero-3-phosphate to diacylglycerophosphate show a very marked specificity for long chain acyl-CoA's. Therefore, we conclude that the predominant localization of long chain fatty acids in the 1 and 2 positions, and of shorter chain fatty acids in the 3 position of the glycerol backbone, results at least in part from the specificities of the mammary gland acyltransferases.

Supplementary key words milk fat triglycerides · stereospecificity · diglyceride:acyl-CoA acyltransferase · noncorrelative acylation · gas–liquid chromatography of radioactive triglycerides

The milk triglycerides of some mammals differ from other tissue triglycerides in that they contain considerable amounts of short or medium chain-length[3] fatty acids (1–3). The distribution of the milk fatty acids on the glycerol backbone is not random, for the long chain acids are found predominantly in positions 1 and 2 and the shorter chain acids are found predominantly in the 3 position of the glycerol (2, 4–6).

The most widely accepted mechanism of milk fat synthesis predicts that fatty acids are transferred to specific positions of the glycerol moiety, but that within those positions they are distributed evenly over all species of triglycerides (6). Implicit in this hypothesis is the concept that fatty acids already esterified to glycerol do not influence the specificity of subsequent acylations with other fatty acids. This hypothesis is usually referred to as the theory of noncorrelative acylation (7).

We have attempted to test this theory by studying the specificity of the acyltransferases involved in milk triglyceride biosynthesis. In a previous communication (8) we demonstrated that in lactating rat mammary gland, the acyltransferases concerned with the biosynthesis of phosphatidic acid showed marked specificity for long chain acyl-CoA substrates. These results were consistent with the observed predominance of long chain fatty acids in the 1 and 2 positions of milk fat triglycerides (2, 4–6). In this communication we report the results of our study on the acyltransferase system responsible for the conversion of di- to triglyceride, with particular attention to the acyl donor and diglyceride acceptor specificities.

MATERIALS

Tritium-labeled acyl-CoA derivatives of high specific radioactivity were prepared from tritiated fatty acids via their *N*-hydroxysuccinimide esters.

Synthesis and purification of tritiated fatty acids

Fatty acids (C_{10}, C_{12}, C_{14}, and C_{18}) were purchased from the Hormel Institute, Austin, Minn., and were tritiated through a silent electric discharge procedure by ICN, Irvine, California. The ^3H-labeled fatty acids (C_{12}, C_{14}, and C_{18}) were dissolved in hexane, and a large proportion of contaminating radioactivity was removed by repeated washing with dilute HCl. The

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[3] The terms short, medium and long chain are used for convenience to indicate fatty acids of 4–6, 8–12, and 12 or more carbon atoms respectively.

organic phases were concentrated and the labeled fatty acids were purified by chromatography on silica Gel G (0.5 mm) plates, using hexane–diethyl ether–acetic acid 60:40:1, (v/v/v) as the developing solvent. ^{3}H-Labeled decanoic acid was purified by a urea column procedure (9).

Synthesis of *N*-hydroxysuccinimide esters of tritiated fatty acids

The esters were prepared by a microadaptation of the method of Lapidot, Rappoport, and Wilman (10). The esters were purified by recrystallization from ethanol, and their purity was confirmed by the observation that their melting points were identical with reported values (10).

Preparation of acyl-CoA derivatives

^{3}H-Labeled acyl-CoA derivatives were prepared from the appropriate *N*-hydroxysuccinimide esters by the procedure of Al-Arif and Blecher (11). The acyl-CoA derivatives (C_{12}, C_{14}, and C_{18}) were purified by acid precipitation and the precipitates were washed with acetone and diethyl ether (12).

^{3}H-Labeled decanoyl-CoA was purified by chromatography on DEAE-cellulose (13). The specific radioactivity of the [^{3}H]decanoyl-CoA and the ratio E 232 nm/E 260 nm (0.57 ± 0.04) was constant in the fractions collected from the column. The fractions containing acyl-CoA were pooled and the NaCl was removed by treatment with Dowex 50W-X8, hydrogen form. The material was lyophilized and redissolved in water.

The overall yield for the synthesis of the tritiated acyl-CoA derivatives was 20–40% with specific activities in the range of 33–62 μCi/μmole.

The [1-^{14}C]palmitoyl-CoA, [1-^{14}C]stearoyl-CoA and [1-^{14}C]oleoyl-CoA were purchased from New England Nuclear Corp., Boston, Mass. Specific activities were approximately 60 μCi/μmole.

Acyl-CoA concentrations were assayed by measuring, with 5,5-dithiobis-(2-nitrobenzoate), the sulfhydryl groups (14) released after mild alkaline hydrolysis, or by measuring the extinction at 232 and 260 nm (15). Acyl-CoA derivatives were stored at pH 6, −70°C.

The enantiomeric diglycerides were purchased from Supelco, Inc., Bellefonte, Pa., and stored in benzene, under nitrogen, at −20°C. This company synthesizes *sn*-1,2- and 2,3-diglycerides from D- and L-mannitol respectively (16) so that the products are true enantiomers. Racemic 1,2- and 1,3-diglycerides were obtained from Nu-Chek Prep, Inc., Elysian, Minn. Solutions of the various diglycerides in benzene were stable for at least three months. Before each experiment, the solvent was removed from a portion of the benzene solution under a stream of nitrogen. The diglycerides were dissolved in acetone at a concentration of 1 mM. The purity of the diglycerides was confirmed periodically by thin-layer chromatography. No significant isomerization could be detected.

The *sn*-[U-^{14}C]glycero-3-phosphate was prepared and purified as described previously (8); [1-^{14}C]-tripalmitin and [1-^{14}C]tristearin were synthesized as described by Calvin et al. (17). Other chemicals, reagents, and cofactors were of the highest purity available.

Milk fat triglycerides were isolated (3) from the milk of lactating rats that were fed a fat-free diet (18) for seven days. These triglycerides were used as carrier for the gas–liquid radiochromatographic analyses.

METHODS

Isolation of subcellular fractions

These fractions were isolated from homogenates of freshly dissected lactating rat mammary glands by differential centrifugation (8). Mitochondrial and microsomal fractions were washed by suspension and recentrifugation in 0.05 M Tris-HCl buffer, pH 7.4, containing 1 mM EDTA. The final pellets were suspended in the same buffer to give a protein concentration of 2 mg/ml. Protein was determined by the method of Gornall, Bardawill, and David (19), using defatted human serum albumin as standard.

Assay system for acyl-CoA diglyceride: acyltransferase

Unless otherwise specified, all reaction mixtures contained in a total volume of 0.5 ml:Tris-HCl buffer, pH 7.4, 50 μmoles; radioactive acyl-CoA, 5 nmoles; defatted human serum albumin, 0.1 mg; diglyceride, 50 nmoles in 0.05 ml of acetone; microsomal protein, 0.02–0.04 mg. The acetone solution of diglyceride was added to the reaction mixture before the addition of enzyme, as described by Ailhaud et al. (20). The reaction was started by the addition of enzyme and allowed to proceed for 2–5 min at 37°C, it was terminated by the addition of 5 ml of a mixture containing petroleum ether (bp 30–60°C)–isopropanol–1 N H_2SO_4 10:40:1 (v/v/v) (21). Two clear phases were obtained after the further addition of 4 ml of petroleum ether and 2 ml of water. A syringe was used to remove the lower phase, and the upper phase was washed once with 5 ml of 0.2 M KCl and twice with 5 ml of 0.2 M $NaHCO_3$. The petroleum ether was evaporated and the lipid was dissolved in

0.5 ml of $CHCl_3$–CH_3OH 2:1 (v/v). In some experiments, the bicarbonate washings were acidified and the fatty acids were extracted with hexane to determine the extent of hydrolysis of acyl-CoA to free fatty acids. An aliquant portion of the chloroform–methanol extract was taken for the determination of radioactivity, and the remainder was applied to a silica gel thin-layer plate.

In early experiments, dithiothreitol was included in the assay system. This practice was discontinued when we found that dithiothreitol had no stimulatory effect on acyltransferase activity; dithiothreitol can also interfere with assays involving acyl-CoA substrates, under certain conditions, due to the formation of *O*-acyl dithiothreitol (22).

Control incubation systems containing no enzyme were run for each acyl-CoA with every experiment. We used "4 or 19 channel linear-Q" silica gel plates obtainable from Quantrum Industries, Fairfield, N. J., since a large volume of sample can be applied rapidly. Quantitative transfer to the plate was assured by rinsing the reaction tube with 0.5 ml of $CHCl_3$–CH_3OH 2:1 (v/v). The plates were developed with either *n*-hexane–diethyl ether–methanol–acetic acid 90:23:3:2 (v/v/v/v) or chloroform–acetone 100:1.5 (v/v). For identification purposes, a mixture of free fatty acid, mono-, di-, and triglycerides, was applied to one channel of the plate and the glycerides were localized by spraying with 2,7-dichlorofluorescein (0.05% methanol) and examination under a UV lamp.

The channels of each chromatogram were divided into narrow horizontal bands and the silica gel was quantitatively transferred to scintillation vials for determination of radioactivity.

Determination of radioactivity

Radioactivity was determined by liquid scintillation spectrometry using 10 ml of a solution of Omnifluor (New England Nuclear Corp.) in toluene–ethoxyethanol 2:1 (v/v). Efficiency was determined by the channels ratio method (23).

Gas–liquid chromatographic analysis of radioactive triglycerides

Triglycerides were isolated by thin-layer chromatography and fractionated on a stainless steel column (3′ × ⅛″) containing 2% Dexsil 300 on Anakrom Q, 60–70 mesh, using a Perkin-Elmer 3920 gas chromatograph. A carrier gas (Ar–CO_2 95:5) flow rate of 20 ml/min was used, and the column temperature was programmed at 1°C/min from 230°C to 350°C. The effluent from the column was split 9:1; the minor effluent was piped to a flame ionization detector and the main effluent to a combustion–reduction furnace maintained at 750°C. The combustion unit, constructed in our laboratory, consisted of two stainless steel tubes linked with Swagelok couplings and packed successively with copper oxide and iron wire. The effluent from the combustion unit was monitored for radioactivity with a gas proportional counter purchased from Searle Analytical, Des Plains, Ill.

We experienced a previously unreported problem, which deserves mention, in adapting our system for radiochromatography of triglycerides. Our chromatograph is equipped with an automatic aluminum capsule injection system (Perkin-Elmer AS-41) that facilitates introduction of the samples in the absence of solvent. Our initial attempts to introduce samples to the column with the autosampler were fraught with difficulties. When injection port temperatures of 350–400°C were used, introduction of capsules containing [1-^{14}C]tripalmitin or [1-^{14}C]tristearin resulted in the recording of large peaks for both mass and radioactivity within 2 min of sample injection. No radioactivity or mass was eluted from the column subsequently. When we changed to syringe injection, using the same injection port temperature, both mass and radioactivity were eluted with retention times more appropriate for triglycerides. The results obtained with the autosampler were suggestive that, at the port temperature used, decomposition of the triglycerides was occurring in the aluminum capsules. When samples were injected at lower port temperatures, smaller mass and radioactivity peaks were observed at the beginning of the chromatogram and peaks were observed with retention times consistent with triglycerides. However, at these lower port temperatures, volatilization of the triglycerides apparently occurs very slowly and results in unacceptably broad peaks. We, therefore, feel that autosamplers employing aluminum capsules are not suitable for high temperature work with triglycerides. All of the results reported in this paper were obtained using the manual syringe injection procedure and an injection port temperature of 400°C. Under these conditions, recovery of radioactivity was quantitative and the responses of both ionization detector and radiomonitor were directly proportional to the amount of sample injected. The efficiency of the gas proportional counter was 75% as determined with [1-^{14}C]tripalmitin and [1-^{14}C]tristearin.

We confined our gas–liquid chromatographic analyses to ^{14}C-labeled triglycerides since when ^{3}H-labeled triglycerides were used, the radiomonitor traces tailed badly despite the fact that normal mass traces were observed. We attribute this problem to absorption of ^{3}H gas to metal surfaces. The situation was not significantly improved by the introduction of hydrogen carrier gas.

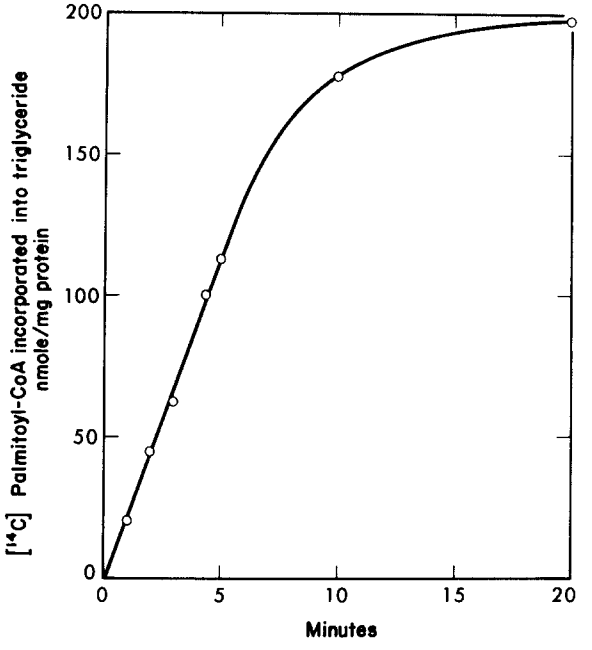

Fig. 1. Time course for acylation of diglyceride. The acyl donor was [1-^{14}C]palmitoyl-CoA (10 μM) and the acceptor, *sn*-1,2-dilaurin (100 μM).

In this report, triglycerides are identified according to the total number of carbon atoms in the acyl moieties, i.e., the glycerol carbons are not counted.

Stereospecific analysis of rat milk triglycerides

Triglycerides were isolated (3) from the milk of lactating rats fed a chow diet. The stereospecific analysis was carried out by the method of Brockerhoff (24). The fatty acid composition at each glycerol position was determined by the direct and indirect methods outlined by Brockerhoff. Average values for the moles percent composition at each glycerol position were then calculated, the standard error for the moles percent composition averaged ±2 moles %. The distribution of each fatty acid among the *sn*-1,2, and 3 positions was derived from the moles percent compositions.

RESULTS

Subcellular localization

Acyl-CoA:1,2-diglyceride acyltransferase activity was localized predominantly in the microsomal fraction of lactating rat mammary gland. Specific activities in mitochondrial, microsomal, and cytosol fractions were 0.58, 5.03, and 0.14 nmoles/min per mg protein, respectively, when *rac*-1,2-dilaurin and [^{3}H]lauryl-CoA were used as substrates. With these same substrates, the reaction proceeded as a linear function of microsomal protein concentration to a level of about 40 μg/0.5 ml.

Time course for appearance of products

The formation of triglycerides proceeded as a linear function of time for about 6 min (**Fig. 1**). In separate experiments we found that the reaction rate was approximately 30% lower in the absence of albumin. The time course of the hydrolysis of acyl-CoA was also studied and we found that albumin inhibited this reaction. The amount of acyl-CoA removed by hydrolysis (about 2%) was small compared to that incorporated into triglyceride. A small amount of labeled diglyceride was also formed (1/30th of the labeled triglyceride), possibly as the result of: (*a*) acylation of endogenous substrate, (*b*) hydrolysis of synthesized triglycerides, or (*c*) an exchange reaction.

Effect of pH

In two separate experiments, diglyceride acyltransferase activity exhibited little dependence on pH between 5.7 and 8.8 when *rac*-1,2-dilaurin and [^{3}H]-lauryl-CoA were used as acyl acceptor and acyl donor, respectively. The results of one experiment are shown in **Fig. 2.**

Acyl-CoA requirement

When *sn*-1,2-dilaurin was used as the acyl acceptor to study the dependence of reaction rate on acyl-CoA concentration, optimal activity was observed at approximately 10 μM for each acyl-CoA tested (**Fig. 3**). The long chain acyl-CoA's, C_{14}, C_{16}, and C_{18}, were better acyl donors than the medium chain acyl-CoA's, C_{10} and C_{12}. Oleoyl-CoA was inferior to stearoyl-CoA. Similar results were obtained in four other experiments using *rac*-1,2-dilaurin as acyl ac-

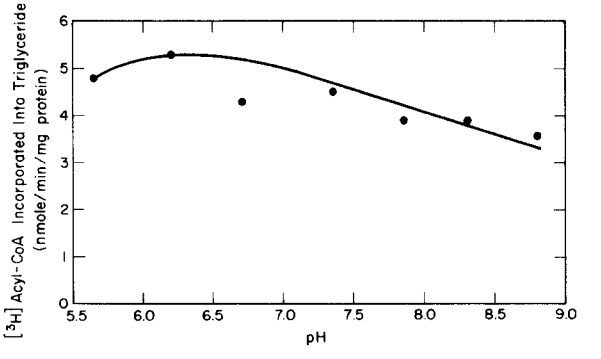

Fig. 2. The effect of pH on the acylation of diglyceride. Tris–maleate (0.1 M) was used as buffer, the acyl donor was [^{3}H]-lauroyl-CoA (10 μM) and the acceptor, *rac*-1,2-dilaurin (100 μM). Dithiothreitol (2 mM) was included in these incubations. The amount of defatted human serum albumin (0.5 mg) used in these assays was higher than that routinely used (0.1 mg) but does not affect the acyltransferase activity significantly.

ceptor. The product synthesized from dilaurin and [1-^{14}C]palmitoyl-CoA was shown by gas–liquid radiochromatography to be exclusively a triglyceride of carbon number C_{40}; similarly, the only product synthesized from dilaurin and [1-^{14}C]stearoyl-CoA was a C_{42} triglyceride. The contribution of endogenous acceptors must have been negligible, otherwise triglycerides of different carbon number would have also been found.

Diglyceride requirement

The dependence of the reaction rate of the concentration of various *sn*-1,2-diglycerides was examined (**Fig. 4**). The best acceptor was *sn*-1,2-dilaurin; longer chain diglycerides were less effective.

The acyltransferase activity exhibited a high degree of stereospecificity (**Table 1**). The *sn*-1,2-enantiomers of dilaurin and dimyristin were much better acceptors than the corresponding *sn*-2,3-enantiomers. The effectiveness of *sn*-2,3-dimyristin as an acceptor for palmitoyl-CoA was virtually unchanged over the range of diglyceride concentration 10–200 μM (not shown in table). Very little activity was observed with *rac*-1,3-dilaurin.

Effect of diglyceride acceptor on acyl-CoA specificity

To test the theory of noncorrelative acylation (7), the effectiveness of the various acyl-CoA's as acyl donors was compared using different diglyceride

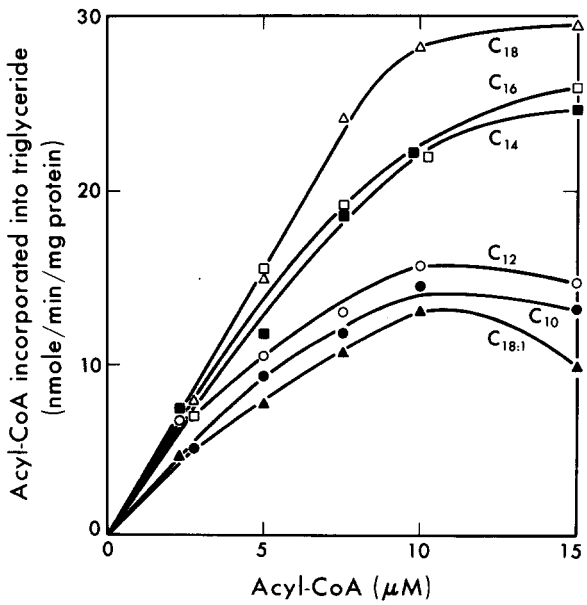

Fig. 3. The effect of acyl-CoA concentration on acylation of diglyceride. The acyl acceptor was *sn*-1,2-dilaurin (100 μM) and the acyl donors were [^{3}H]decanoyl-CoA, [^{3}H]lauroyl-CoA, [^{3}H]myristoyl-CoA, [1-^{14}C]palmitoyl-CoA, [1-^{14}C]stearoyl-CoA and [1-^{14}C]oleoyl-CoA.

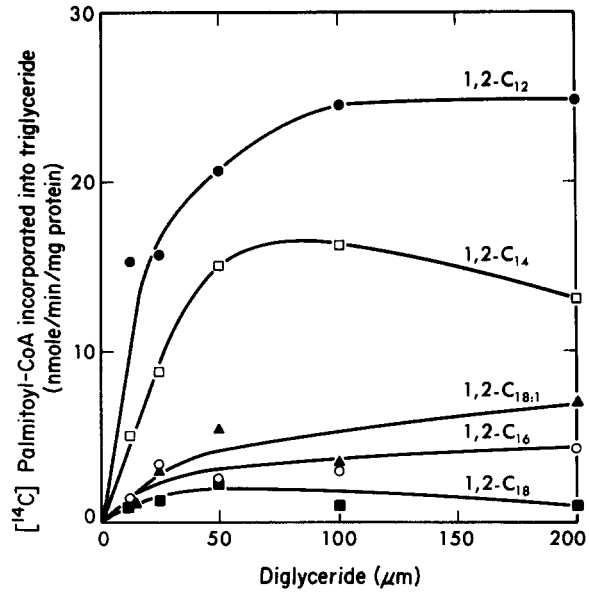

Fig. 4. The effect of diglyceride concentration on acyltransferase activity. The acyl donor was [1-^{14}C]palmitoyl-CoA (10 μM) and all diglycerides used were the *sn*-1,2 enantiomers.

acceptors (**Table 2**). Only a small amount of triglyceride was formed in the absence of added diglyceride, presumably due to the presence of endogenous diglycerides. All *sn*-1,2-diglycerides increased the incorporation of acyl-CoA into triglycerides. The most effective acceptors, *sn*-1,2-dilaurin and *sn*-1,2-dimyristin, stimulated the reaction 15- to 55-fold over the level observed in the absence of added diglyceride. The order of preference for *sn*-1,2-diglycerides with all acyl-CoA donors was dilaurin > dimyristin > dipalmitin > distearin. The effectiveness of *sn*-1,2-diolein was intermediate between that of dipalmitin and distearin for most of the acyl-CoA donors.

The acyl specificity was very broad and was more or less independent of the *sn*-1,2-diglyceride acceptor. Similar results were obtained in four other experiments when *rac*-1,2-diglycerides were the acceptors.

The observed preference of the acyltransferase system for medium chain diglyceride acceptors was rather unexpected in view of the fact that these diglycerides are much less likely to be synthesized from *sn*-glycerol-3-phosphate than are long chain diglycerides (8).

In view of the problems inherent in preparing lipid substrates, we considered the possibility that the more hydrophobic diglycerides might not be presented to the enzyme in a suitable physical state. We therefore investigated various techniques for emulsifying *rac*-1,2-dipalmitin such as sonication, addition of detergents, and various organic solvents. None of

TABLE 1. Stereospecificity of diglyceride acyltransferase activity

Diglyceride	Palmitoyl-CoA Converted to Triglyceride
	nmoles/min/mg protein
None	0.18 ± 0.04 (6)
sn-1,2-dilaurin	19.4 ± 2.4 (9)
sn-2,3-dilaurin	0.91 ± 0.01 (3)
rac-1,3-dilaurin	0.25 ± 0.03 (3)
sn-1,2-dimyristin	10.2 ± 2.3 (4)
sn-2,3-dimyristin	0.95 ± 0.05 (2)

The acyl donor was [1-^{14}C]palmitoyl-CoA (10 μM). The results are presented as the average ± SE with the number of animals in parentheses. The activity observed in the absence of added diglyceride has not been subtracted from the activity observed in the presence of the various diglycerides.

these methods was more effective than our routine assay procedure.

In a further attempt to resolve this problem, we made use of the technique of gas–liquid chromatography of triglycerides to examine the products formed when various diglycerides were presented to the enzyme system as mixed micelles (**Fig. 5**). The *sn*-1,2-diglyceride that provided the acceptor for a particular acyl moiety could be identified simply by subtracting the number of carbon atoms in the labeled acyl-CoA from the carbon number of the labeled triglyceride product. Thus when [1-^{14}C]palmitoyl-CoA was the acyl donor, C_{40} triglyceride was the major product, therefore C_{24} diglyceride, i.e., *sn*-1,2-dilaurin, must have been the preferred acceptor (**Table 3**). When the mixture of *sn*-1,2-dilaurin, dimyristin, dipalmitin, and distearin (LMPS) was used, the order of preference of diglyceride acceptors was dilaurin > dimyristin > dipalmitin > distearin (Table 3); this was the same order of preference found when the diglycerides were compared in separate incubations (Table 2).

TABLE 2. Effect of various *sn*-1,2-diglycerides on the acyl-CoA specificity of the acyltransferase reaction

Acyl-CoA	Acyltransferase Activity with Specified Acceptors					
	None	Dilaurin	Dimyristin	Dipalmitin	Distearin	Diolein
	nmoles acyl-CoA incorporated/min/mg protein					
C_{10}	0.1	5.5	2.7	1.0	0.3	1.7
C_{12}	0.2	4.5	3.0	1.3	0.6	0.9
C_{14}	0.2	6.3	5.0	2.8	0.8	1.9
C_{16}	0.2	7.1	5.3	2.6	0.8	2.6
C_{18}	0.2	7.9	5.6	1.8	0.9	1.3
$C_{18:1}$	0.1	5.5	4.3	1.6	0.4	1.6

Acyl-CoA concentration was 10 μM and diglyceride concentrations 100 μM. Results are the mean of two experiments performed with microsomal fractions from different rats. The small contribution of endogenous acceptors to acyltransferase activity, shown in the first column, has not been subtracted from the activities reported in the presence of added diglycerides.

When the mixture of *sn*-1,2-dilaurin, dimyristin, dipalmitin, and diolein (LMPO) was used, again the order of preference for the saturated diglyceride acceptors was dilaurin > dimyristin > dipalmitin; the effectiveness of diolein was intermediate between that of dipalmitin and distearin (Table 3), as was the case when each diglyceride was incubated separately (Table 2).

Stereospecific distribution of fatty acids in rat milk triglycerides

From consideration of the fatty acid composition and the molecular weight distribution of rat milk triglycerides, Breach, Dils, and Watts (6) suggested that the arrangement of fatty acids on the glycerol backbone was nonrandom. This prediction is confirmed by our stereospecific analysis (**Fig. 6**). The proportion of each fatty acid in the *sn*-3 position decreased with increasing chain length from C_8 to C_{16}. Conversely, the proportion of these acids in the *sn*-2 position increased with increasing chain length. Thus the medium chain fatty acids C_8, C_{10}, and C_{12} tend to favor the *sn*-3 position, C_{14} and C_{16} favor the *sn*-1 position, and fatty acids with 18 carbon atoms favor the *sn*-1 position.

DISCUSSION

Hanwell and Linzell (25) have measured the daily output of milk in the laboratory rat and obtained values of 25 and 42 g/day for dams nursing 6 and 12 pups, respectively. With our microsomal system, we have observed a mean activity of 19.4 ± 2.4 nmoles (standard error for nine experiments) of palmitoyl-CoA transferred to *sn*-1,2-dilaurin per min per mg protein at 37°C. Given: (*i*) the triglyceride content of rat milk is approximately 10% by weight[4], (*ii*) an average triglyceride molecular weight of 720 (3), (*iii*) the microsomal protein content of rat mammary gland is 10 mg/g wet weight[5], (*iv*) the average weight[6] of the mammary glands of a lactating rat is 15 g, we calculate that our microsomal system synthesizes the equivalent of 3.0 g of triglyceride per day. This would be equivalent to about 30 g of milk, a value within the physiological range measured by Hanwell and Linzell. We conclude therefore, that our in vitro system represents an adequate model for the in vivo situation.

The fatty acid component of milk fat triglycerides is derived from two sources, blood lipids and fatty

[4] Mozes N., unpublished results.
[5] Lin, C. Y., unpublished results.
[6] Abraham S., unpublished results.

acids synthesized within the mammary gland epithelial cells (for recent reviews, see ref. 26–29). In most species, blood lipids in the form of chylomicron triglycerides are transported to the mammary gland where they are hydrolyzed by the lipoprotein lipase from the capillary endothelial cells. The long chain fatty acids produced in the reaction are transferred to the mammary gland epithelial cells (28). All of the shorter chain fatty acids and some of the long chain fatty acids are synthesized within the mammary gland epithelial cells (30, 31).

Stereochemical analyses of the milk fat triglycerides of several species have shown that the fatty acids in positions 1 and 2 of the glycerol moiety are predominantly the long chain fatty acids derived from blood lipids, whereas the locally synthesized shorter chain fatty acids are found preferentially esterified to position 3 (2, 4–6, 32). The results presented here (Fig. 6) indicate this situation to be true also in the case of rat milk triglycerides. Although a previous study has been made on the acyltransferase specificity of goat mammary gland (33), until now no data have been obtained that provide an explanation for the observed distribution of fatty acids in milk triglycerides.

The results of our earlier work provided a rationalization, in terms of acyltransferase specificity, for the predominance of long chain fatty acids in positions 1 and 2 (8). The data reported here provide a possible explanation for the presence of medium chain fatty acids preferentially in position 3. The microsomal acyltransferases responsible for the biosynthesis of phosphatidic acid, i.e., the enzymes acylating positions 1 and 2, are highly specific for long chain fatty acids; palmitoyl-CoA is almost eighteen times more effective as an acyl donor than is decanoyl-CoA (8). However, the microsomal enzyme acylating diglycerides shows a much broader acyl specificity, e.g., palmitoyl-CoA is only about twice as effective as decanoyl-CoA as an acyl donor. Thus, there is a much greater probability of the medium chain fatty acids being esterified to position 3 rather than to positions 1 and 2.

Our stereospecific analysis of rat milk triglycerides indicated that myristic and palmitic acids were preferentially esterified to the *sn*-2 position. A preferential association of myristic acid with the *sn*-2 position also has been reported for cow (34), sheep (5), goat (5), and monkey (35) milk triglycerides. At present, we cannot say whether this phenomenon is due to different specificities of the acyltransferases acylating the *sn*-1 and *sn*-2 positions, since our previous experiments (8) did not distinguish between these two acyltransferases. The question could possibly be resolved by using the acyl acceptors 1-acylglycerophosphate and 2-acylglycerophosphate, as did Yamashita, Hosaka and Numa (36) in their studies on the acyltransferase system of rat liver microsomes.

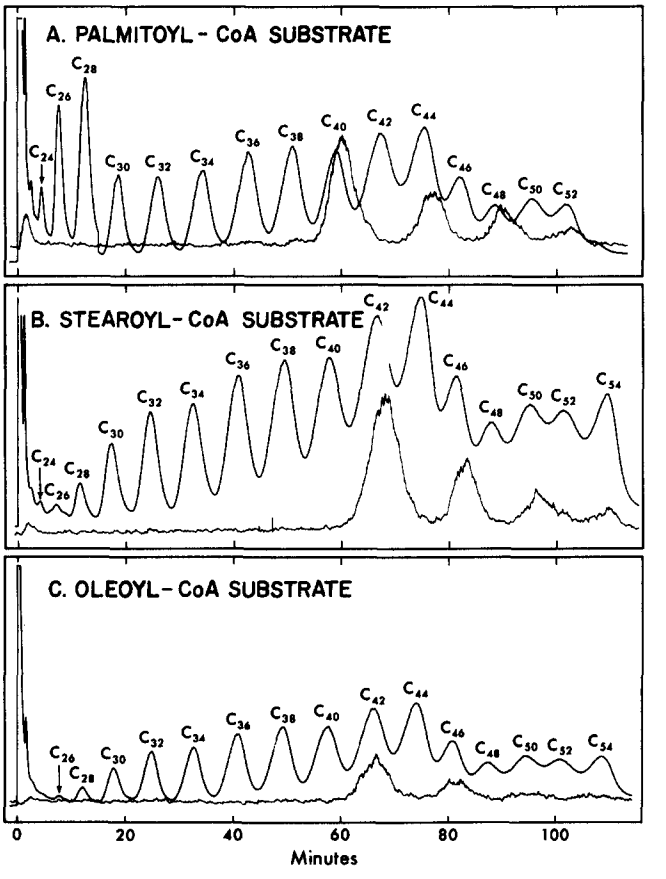

Fig. 5. Gas chromatograms of radioactive triglycerides synthesized from mixed diglyceride acceptors and various acyl-CoA donors. The diglyceride acceptor mixture contained 25 μM each of *sn*-1,2-dilaurin, dimyristin, dipalmitin, and diolein, and the acyl donors were *A*. [1-^{14}C]palmitoyl-CoA (10 μM), *B*. [1-^{14}C]stearoyl-CoA (10 μM), and *C*. [1-^{14}C]oleoyl-CoA (10 μM). The procedure for gas–liquid chromatography is described in the Methods section. The carrier triglyceride mixture used in A was ~0.5 mg of rat milk triglycerides; in B and C, 50 μg of tristearin was added to the milk triglyceride carrier. The flame ionization attenuations were *A*) X16, changed to X32 after 15 min, *B*) X16, *C*) X32. Full scale on the radiomonitor recording (the lower trace) was 1500 cpm. The slight displacement to the right of the radiomonitor tracings is caused by the time lapse between the sample reaching the ionization detector and the gas proportional counter. In cases where the radioactive triglyceride species contained an unsaturated fatty acid, there was a much shorter lag time between the appearance of the mass and radioactive peaks. This is due to the fact that, whereas the column separates triglycerides primarily according to carbon number, unsaturated triglycerides are eluted slightly before saturated triglycerides of the same carbon number.

In our previous studies (8), we found that the major product formed by acylation of glycerophosphate was phosphatidate with only small amounts of neutral glyceride. The present study demonstrates that these microsomal preparations have a high

TABLE 3. Effect of mixed *sn*-1,2-diglyceride acceptors on diglyceride acyltransferase specificity

Donor Acyl-CoA	Acceptor Diglyceride Mixture	Acyl-CoA Incorporated into Triglycerides of Specified Carbon Number								
		Total	40	42	44	46	48	50	52	54
		nmoles/min/mg protein								
16	LMPO	13.9	6.91		3.61		2.14		1.24	
18	LMPO	12.9		6.83		3.01		1.62		0.73
18	LMPS	12.8		9.11		2.33		1.08		0.28
18:1	LMPO	5.0		2.60		1.27		0.69		0.45
18:1	LMPS	8.5		5.29		2.26		0.66		0.30

The diglyceride acceptor mixture LMPO consisted of 25 μM each of *sn*-1,2-dilaurin, dimyristin, dipalmitin, and diolein; the diglyceride mixture LMPS consisted of 25 μM each of *sn*-1,2-dilaurin, dimyristin, dipalmitin, and distearin. The incorporation into individual triglycerides was calculated from the gas–liquid radiochromatograms; the chromatograms obtained with the diglyceride mixture LMPO are shown in Fig. 5. Note that the triglycerides are identified only by carbon number, no distinction is made between saturated and unsaturated glycerides. The exact nature of the triglyceride can, however, be inferred from consideration of the available donor and acceptors, thus, the C_{54} triglyceride synthesized from stearoyl-CoA and the LMPO diglyceride mixture must be 18, 18:1, 18:1 whereas the C_{54} triglyceride synthesized from stearoyl-CoA and the LMPS diglyceride mixture must be 18, 18, 18.

capacity for converting diglyceride to triglyceride. It appears, therefore, that the rate limiting step in triglyceride synthesis by microsomal preparations is the hydrolysis of phosphatidate to diglyceride. Several workers have shown that addition of a cytosol fraction to the microsomal fraction will greatly accelerate the conversion of phosphatidate to neutral glyceride and have ascribed this effect to the presence of a phosphatidate phosphohydrolase in this subcellular fraction (37). We have also found that addition of a cytosol fraction to the microsomal fraction from lactating rat mammary gland will greatly stimulate the conversion of phosphatidate to di- and triglyceride. Whether the effect is due to a phosphatidate phosphohydrolase or to some other factors (38, 39) is not certain. However, it is clear that in order to convert glycerophosphate to triglyceride efficiently, participation by both microsomal and cytosol fractions is required.

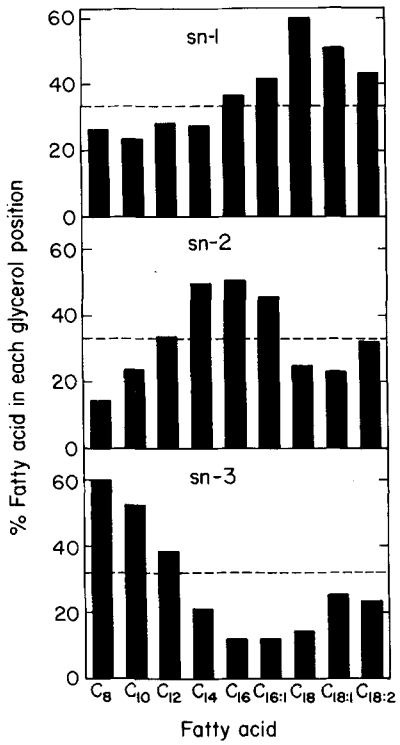

Fig. 6. Stereospecific distribution of fatty acids in rat milk triglycerides. The dotted line at 33.3% indicates the proportion of each fatty acid expected on the basis of a random distribution.

The second facet of milk triglyceride synthesis we investigated concerned the question of specificity of the acyltransferase for diglyceride acceptor. The specificity of the acyltransferase for diglycerides is highly selective as far as the stereospecific position of the acyl groups on the glycerol moiety is concerned. The *sn*-1,2-enantiomers are clearly the preferred species. This observation is consistent with the fact that the *sn*-1,2-diglycerides are the natural intermediates in triglyceride biosynthesis. O'Doherty, Kuksis, and Buchnea (40) also found that *sn*-1,2-diglycerides were better acyl acceptors than *sn*-2,3-diglycerides in microsomal preparations from rat liver and intestine, although the difference in effectiveness was not nearly so marked as we have found with the lactating rat mammary gland system. It should be pointed out, however, that whereas O'Doherty et al. (40) compared *sn*-1,2- and *sn*-2,3-diglycerides with different substituent acyl groups (*sn*-1-stearoyl-, 2-linolenoyl- versus *sn*-2-palmitoyl-, 3-oleoylglycerols) we compared enantiomers with

identical substituent acyl groups (*sn*-1,2-dilaurin versus *sn*-2,3-dilaurin and *sn*-1,2-dimyristin versus *sn*-2,3-dimyristin).

The 1,3-diglycerides appear to be poorly utilized by microsomal preparations from most of the tissues studied, i.e., chicken adipose tissue (41), lactating goat mammary gland (33), rat liver and intestine (40), and lactating rat mammary gland (this study). Akesson (42), however, found that a pig liver microsomal fraction utilized 1,3-diglyceride as much as 50% as effectively as *rac*-1,2-diglyceride. Whether in this case the 1,3-diglyceride was utilized directly or underwent partial hydrolysis prior to acylation, as suggested by O'Doherty et al. (40), is not clear at present. With the lactating rat mammary gland system, we found that the specificity for the constituent acyl groups is rather broad, although the effectiveness as acyl acceptor decreases somewhat with increasing chain length.

With lipid substrates, such as diglycerides, there is always some doubt as to whether the physical state of the substrate offered to the enzyme might influence markedly the observed activity. However, when the more hydrophobic, long chain diglycerides are presented to the enzyme in mixed micelles with less hydrophobic species, the shorter chain diglycerides were always the preferred acceptors. Thus, whether the diglycerides were present singly or together, the order of preference was the same, i.e., dilaurin > dimyristin > dipalmitin > distearin. While we have gone to considerable lengths to evaluate the possible influence of the physical form of the diglyceride substrates on their effectiveness as acyl acceptors—we have compared the effects of various detergents and solvents, sonication, mixed micelles, etc.—we cannot say unequivocally that the apparent preference for shorter chain diglycerides does not result at least partially from the greater "solubility" of these substrates.

The theory of "noncorrelative acylation" implies that the type of fatty acid introduced into the glycerol backbone should not influence the specificity of subsequent acylation steps. The results of our experiments support this hypothesis, for we observed essentially no change in the acyl specificity when different diglycerides were used as acceptors.

In conclusion, our results support the theory that the nonrandom arrangement of fatty acids in milk triglycerides results, at least in part, from the specificities of the mammary gland acyltransferases.

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REFERENCES

1. Brockerhof, H., R. J. Hoyle, and N. Wolmark. 1966. Positional distribution of fatty acids in triglycerides of animal depot fats. *Biochim. Biophys. Acta.* **116:** 67–72.
2. Breckenridge, W. C., and A. Kuksis. 1967. Molecular weight distribution of milk fat triglycerides from seven species. *J. Lipid Res.* **8:** 473–478.
3. Smith, S., R. Watts, and R. Dils. 1968. Quantitative gas–liquid chromatographic analysis of rodent milk triglycerides. *J. Lipid Res.* **9:** 52–57.
4. Brockerhof, H. 1971. Stereospecific analysis of triglycerides. *Lipids.* **6:** 942–956.
5. Kuksis, A., L. Marai, and J. J. Myher. 1973. Triglyceride structure of milk fats. *J. Amer. Oil Chem. Soc.* **50:** 193–201.
6. Breach, R. A., R. Dils, and R. Watts. 1973. Milk triglycerides: the degree of nonrandom associations of fatty acids. *J. Dairy Res.* **40:** 273–287.
7. Slakey, P. M., and W. E. M. Lands. 1968. The structure of rat liver triglycerides. *Lipids.* **3:** 30–36.
8. Tanioka, H., C. Y. Lin, S. Smith, and S. Abraham. 1974. Acyl specificity in glyceride synthesis by lactating rat mammary gland. *Lipids.* **9:** 229–234.
9. Kumar, S., V. N. Singh, and R. Keren-Paz. 1965. Biosynthesis of short chain fatty acids in lactating mammary supernatant. *Biochim. Biophys. Acta.* **98:** 221–229.
10. Lapidot, Y., S. Rappoport, and Y. Wilman. 1967. Use of esters of *N*-hydroxysuccinimide in synthesis of *N*-acylamino acids. *J. Lipid Res.* **8:** 142–145.
11. Al-Arif, A., and M. Blecher. 1969. Synthesis of fatty acid Coenzyme A and other thiol esters using *N*-hydroxysuccinimide esters of fatty acids. *J. Lipid Res* **10:** 344–345.
12. Seubert, W. 1960. S-palmitoyl Coenzyme A. *Biochem. Prep.* **7:** 80–83.
13. Smith, S., and S. Abraham. 1975. Fatty acid synthase from lactating rat mammary gland. *In* Methods in Enzymology, Vol. 35, J. M. Lowenstein, editor. Academic Press, New York. 65–74.
14. Ellman, G. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82:** 70–77.
15. Stadtman, E. R. 1957. Preparation and assay of acyl-CoA and other thiol esters; use of hydroxylamine. *In* Methods in Enzymology, Vol. 3. S. P. Colowick and N. O. Kaplan, editors. Academic Press, New York. 931–941.
16. Baer, E., and V. Mahadevan. Synthesis of L-α-lecithins containing shorter chain fatty acids. Water-soluble glycerophosphatides. *J. Amer. Chem. Soc.* **81:** 2494–2498.
17. Calvin, M., C. Heidelberger, J. C. Reid, B. M. Tolbert, and P. F. Yankwich. 1949. Isotopic carbon. John Wiley and Sons, New York, 182–183.
18. Smith, S., H. T. Gagné, R. Pitelka, and S. Abraham. 1969. The effect of dietary fat on lipogenesis in mammary gland and liver from lactating and virgin mice. *Biochem. J.* **115:** 807–815.

19. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177:** 751–766.
20. Ailhaud, G., D. Samuel, M. Lazdunski, and P. Desnuelle. 1964. Quelques observations sur le mode d'action de la monoglyceride transacylase et de la diglycéride transacylase de la nuqueuse intestinale. *Biochim. Biophys. Acta.* **84:** 643–664.
21. Yamashita, S., K. Hosaka, M. Taketo, and S. Numa. 1973. Distribution of glycerolipid-synthesizing enzymes in the subfractions of rat liver microsomes. *FEBS Lett.* **29:** 235–238.
22. Stokes, G. B., and P. K. Stumpf. 1974. Fat metabolism in higher plants. The nonenzymatic acylation of dithiothreitol by acyl-CoA. *Arch. Biochem. Biophys.* **162:** 638–648.
23. Baillie, L. A. 1963. Determination of liquid scintillation counting efficiency by pulse-height shift. *In* Adv. in Tracer Methodology. S. Rothchild, editor. Plenum Press, New York. 86–92.
24. Brockerhof, M. 1965. Stereospecific analysis of triglycerides: an analysis of human depot fat. *Arch. Biochem. Biophys.* **110:** 586–592.
25. Hanwell, A., and J. L. Linzell. 1972. A simple technique for measuring the rate of milk secretion in the rat. *Comp. Biochem. Physiol.* **43A:** 259–270.
26. Patton, S. 1973. Origin of the milk fat globule. *J. Amer. Oil Chem. Soc.* **50:** 178–185.
27. Patton, S., and R. G. Jensen. 1975. Lipid metabolism and membrane functions of the mammary gland. *In* Progress in the Chemistry of Fats and Other Lipids, Vol. 14, part 4. R. T. Holman, editor. Pergamon Press, Oxford. 163–277.
28. Scow, R. O., C. L. Mendelson, O. Zinder, M. Hamosh, and E. Blanchette-Mackie. 1973. Role of lipoprotein lipase in the delivery of dietary fatty acids to lactating mammary tissue. *In* Dietary Lipids and Postnatal Development. R. Paoletti and C. Galli, editors. Raven Press, New York. 91–114.
29. Smith, S., and S. Abraham. 1975. The composition and biosynthesis of milk fat. *In* Advances in Lipid Research, Vol. 13. R. Paoletti and D. Kritchevsky, editors. Academic Press, New York. 195–239.
30. Abraham, S., P. R. Kerkof, and S. Smith. 1972. Characteristics of cells dissociated from mouse mammary glands. II. Metabolic and enzymatic activities of parenchymal cells from lactating glands. *Biochim. Biophys. Acta.* **261:** 205–218.
31. Martin, R. J., and Baldwin, R. L. 1971. Effects of insulin on isolated rat mammary cell metabolism. *Endocrinology.* **89:** 1263–1269.
32. Kinsella, J. E. 1971. Position of endogenous radioactive fatty acids in mammary triglycerides. *J. Dairy Sci.* **54:** 1014–1017.
33. Pynadath, T. J., and S. Kumar. 1964. Incorporation of short and long chain fatty acids into glycerides by lactating goat mammary tissue. *Biochim. Biophys. Acta.* **84:** 251–263.
34. Pitas, R. E., J. Sampugna, and R. G. Jensen. 1967. Triglyceride structure of cow's milk fat. 1. Preliminary observation on the fatty acid composition of positions 1, 2, and 3. *J. Dairy Sci.* **50:** 1332–1340.
35. Smith, L. M., and S. Mardio. 1974. Intramolecular fatty acid distribution in milk triglycerides of monkeys. *Lipids.* **9:** 713–716.
36. Yamashita, S., K. Hosaka, and S. Numa. 1973. Acyl donor specificities of partially purified 1-acylglycerophosphate acyltransferase, 2-acylglycerophosphate acyltransferase and 1-acylglycerophosphorylcholine acyltransferase from rat liver microsomes. *Eur. J. Biochem.* **38:** 25–31.
37. Hubscher, G. 1970. Glyceride metabolism. *In* Lipid Metabolism. S. J. Wakil, editor. Academic Press, New York. 279–370.
38. O'Doherty, P. J. A., and A. Kuksis. 1975. Stimulation of triacylglycerol synthesis by Z protein in rat liver and intestinal mucosa. *FEBS Lett.* **60:** 256–258.
39. Roncari, D. A. K., and E. Y. W. Mack. 1976. Characterization of a liver cytosolic compound which stimulates triglyceride biosynthesis. *Federation Proc.* **35:** Abstr. 1358.
40. O'Doherty, P. J. A., A. Kuksis, and D. Buchnea. 1972. Enantiomeric diglycerides as stereospecific probes in triglyceride synthesis in vitro. *Can. J. Biochem.* **50:** 881–887.
41. Goldman, P., and P. R. Vagelos. 1961. The specificity of triglyceride synthesis from diglycerides in chicken adipose tissue. *J. Biol. Chem.* **236:** 2620–2623.
42. Akesson, B. 1969. The acylation of diacylglycerols in pig liver. *Eur. J. Biochem.* **9:** 406–414.