# Acceptors of fatty acid for glyceride synthesis in guinea pig mammary gland

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SUMMARY Glycerides are shown to be synthesized by two pathways in mammary tissue of lactating guinea pigs. One pathway involves the direct acylation of monoglycerides. The other pathway proceeds from the acylation of α-glycerophosphate through L-α-phosphatidic acids and D-α,β-diglycerides. Ethanol is also esterified by homogenates of lactating mammary gland.

TWO PATHWAYS have now been described for the biosynthesis of glycerides in mammalian tissues. In the pathway which exists in liver (1, 2) and adipose tissue (3), triglycerides are synthesized from L-α-glycerophosphate via L-α-phosphatidic acids and D-α,β-diglycerides. More recently it has been found that a second pathway involving the direct acylation of monoglyceride exists in intestinal mucosa (4–7), kidney (8), liver (8), and aorta (9).

During the course of our studies on fat metabolism in the lactating mammary gland (10–12), it became necessary to investigate the pathways of biosynthesis of glycerides in this tissue. Evidence will be presented in this paper for the existence in lactating mammary tissue of both known pathways of glyceride synthesis. Part of this work was presented at the April 1963 meeting of the Federation of American Societies for Experimental Biology (13).

## MATERIALS AND METHODS

Mammary glands were removed from lactating N.I.H. strain guinea pigs which were approximately 1 week post partum. Homogenates were prepared as described in the preceding paper (11).

### *Purification of Radioactive Palmitic Acid*

Palmitic acid-1-$C^{14}$ (29.7 μc/μmole) was obtained from the Nuclear-Chicago Corporation, Chicago, Ill. It was dissolved in 50 ml of heptane–glacial acetic acid 1:1 (v/v), 5 ml of water were added, and the lower phase was discarded. The heptane phase was washed three times with 0.02 N $H_2SO_4$ to remove any short-chain fatty acids that might have been present. The palmitic acid was then partitioned in heptane–isopropanol–water–1 N NaOH 40:40:30:1 to remove any neutral contaminants, and the palmitic acid-1-$C^{14}$ was extracted from the aqueous isopropanol phase with heptane after acidification. After evaporation of the heptane under a stream of nitrogen at room temperature, the free fatty acid was dissolved in dilute NaOH (final pH approximately 8.0) with heating, to obtain a relatively stable emulsion. Aliquots were added to carrier palmitate, esterified with methanol, and analyzed by gas–liquid chromatography with simultaneous recording of mass and radioactivity (14). Methyl palmitate contained 99% of the radioactivity, the remainder of the radioactivity being in methyl myristate.

### *Acceptors*

Triglyceride was isolated from olive oil by chromatography on silicic acid columns (15). 1,2-Diglyceride was prepared by the incubation of triglyceride with pancreatic lipase (16), and isolated by silicic acid column chromatography. 1,3-Diglyceride was prepared from an isomeric mixture of dioleins by repeated recrystallizations from petroleum ether at −20°. Monoolein was purchased commercially and purified by silicic acid chromatography. As there was ample opportunity for isomerization, the monoglyceride probably consisted of the random mixture (88% α, 12% β). Any residual free fatty acids were removed from the glycerides by adding Dowex-2 ($OH^-$) to a solution of the glyceride in diethyl ether saturated with water. The fatty acids could not be extracted with alkaline isopropanol because this resulted in isomerization of the diglycerides.

Phosphatidic acid was prepared by the action of carrot chromoplasts on egg yolk lecithin by the method of Kates (17). The phosphatidic acid was purified by column chromatography on silicic acid.

The purity of each of these materials was assayed by thin-layer chromatography (TLC). According to this method, the monoolein and triglyceride were pure (contamination estimated to be less than 0.25%). 1,2-Diglyceride contained approximately 5% of the 1,3-isomer, and 1,3-diglyceride contained about 1% of the 1,2-isomer. The phosphatidic acid streaked badly on thin-layer chromatograms but had an ester to phosphorus ratio of 1:9, and contained no nitrogen.

All these acceptors posed experimental problems because of their insolubility in water. It was found that adequate, but by no means ideal, aqueous emulsions could be obtained by sonication of the substrates in water immediately prior to use. This was the method of preparation employed in most experiments. In one series of experiments, 5 mg of Tween 80 (polyoxyethylene sorbitan monooleate) per ml of water was added prior to sonication. Only slightly better emulsions were obtained. All acceptor emulsions were examined in the gross, and by darkfield microscopy. Phosphatidic acid gave the best emulsions, with a particle size of less than 1 $\mu$. Triglyceride, 1,2- and 1,3-diglyceride, and monoolein gave satisfactory emulsions with particle sizes ranging from less than 1 to 5$\mu$.

### *Cofactors*

ATP and CoA were products of Pabst Laboratories, Milwaukee, Wis. ATP and CoA solutions were quantified by measurement of absorbancy. $\alpha$-Glycerophosphate was the product of Mann Research Laboratories, New York, N.Y. $\beta$-Glycerophosphate was the product of Eastman Kodak Company, Rochester, N.Y. All other materials were reagent grade.

### *Thin-Layer Chromatography*

Procedures similar to those described by Mangold (18) were used with Silica Gel G (Brinkman Instruments Company, New York, N.Y.) as support. Neutral lipids were chromatographed in a solvent system of heptane–diethyl ether–glacial acetic acid 70:30:1 (v/v/v). In this system, cholesterol esters containing a long-chain fatty acid, triglycerides, free fatty acids, 1,3-diglycerides, 1,2-diglycerides, and monoglycerides were all well separated from each other, but there was sufficient variation in the absolute mobilities from one plate to another to require the use of standards on each plate. Spots were developed with iodine vapor. Phospholipids were run in a solvent system of chloroform–methanol–water 60:35:3. Neutral lipids all migrated with the solvent front in this system except for some slight streaking by free fatty acids. Ammonia was not added, as suggested by Skidmore and Entenman (19), since it caused considerably more streaking of the free fatty acids.

### *Assay of Glyceride Synthesis from Palmitate-1-$C^{14}$*

The incubation mixture contained the following concentrations of: ATP, 3.5 mM; CoA, 0.2 mM; $MgCl_2$, 1 mM; NaF, 0.025 M; Tris buffer pH 7.4, 0.075 M; Na palmitate-1-$C^{14}$, 0.1–0.15 mM; acceptor as indicated; and varying quantities of homogenate in a total volume of 2.0 ml. Samples were incubated for 1 hr at 37° on a Dubnoff shaker, and incubations were terminated by partitioning with aqueous alkaline isopropanol–heptane, which also served to remove free fatty acids as sodium soaps (11). The heptane phase was re-extracted three times with fresh lower phase solvent. In control experiments, without homogenate, less than 0.1% of the radioactivity remained in the heptane phase. This procedure results in the quantitative recovery of triglycerides and diglycerides of long-chain fatty acids ($\geq C_{16}$), but most of the monoglycerides and phosphatides are lost (20). Aliquots of the heptane phase were assayed for radioactivity in a scintillation counter. Corrections were made for quenching as previously described (11). Counting efficiency was approximately 80%. Aliquots of the heptane solution were also assayed by thin-layer chromatography in most instances.

## RESULTS

### *Requirements for Incorporation of Palmitate-1-$C^{14}$ into Glycerides*

Palmitate-1-$C^{14}$ was incorporated into glycerides in the presence of cofactors and homogenate of lactating mammary glands (Table 1). There was an absolute requirement for added ATP, CoA, and $\alpha$-glycerophosphate. The assay system was quite sensitive to the quantity of added ATP, particularly when the 100,000 × $g$ sediment rather than the whole homogenate was used. ATP at 0.25 mM resulted in some stimulation, but approximately 2.5 mM was required for optimal incorporation, and above 3.5 mM there began to be some inhibition. This inhibition by higher concentrations of ATP could be partially reversed by increasing the $Mg^{++}$ concentration. There was a rather broad optimum (1–3 mM) for $Mg^{++}$ concentration. There was some stimulation of incorporation with as little as 0.2 mM $\alpha$-glycerophosphate as acceptor. $\beta$-Glycerophosphate was unable to replace $\alpha$-glycerophosphate. Glycerol (not shown in Table 1) was not as effective as $\alpha$-glycerophosphate, but at high levels did stimulate incorporation over the endogenous level.

### *Intracellular Location of Glyceride-Synthesizing Activity*

Homogenates of lactating mammary gland were fractionated by centrifugation as described in the preceding paper (11). Virtually all the glyceride-synthesizing activity was found in the sediment obtained after centrifugation at 100,000 × *g*. In another experiment in which a 12,000 × *g* (10 min) sediment was also obtained, the activity appeared to be distributed roughly equally between the 12,000 and 100,000 × *g* sediments, but only about 50% of the activity was recovered in that experiment.

### *Acceptors of Palmitate-1-C$^{14}$*

Preliminary results were consistent with the pathway for glyceride synthesis proceeding from L-α-glycerophosphate. Therefore, incubations with various intermediates in that pathway, and closely related compounds, were performed. Representative results are shown in Table 2. The addition of 1,2-diglyceride resulted in a 6-fold stimulation of palmitate incorporation over the endogenous level, whereas 1,3-diglyceride provided much less stimulation. Monoolein caused approximately a 3-fold stimulation. Phosphatidic acid, which formed a very good, almost clear, emulsion, was always extremely effective in stimulating the incorporation of palmitate. The effect of adding triglyceride was rather variable. Triglyceride almost always stimulated to some extent and occasionally, as in Table 2, it was as effective an acceptor as 1,2-diglyceride.

The fact that triglyceride sometimes gave as much stimulation as 1,2-diglyceride suggested the possibility that stimulation by all the acceptors of the incorporation of palmitate-1-C$^{14}$ might be the result of a lipase-catalyzed exchange reaction rather than the synthesis of new ester bonds. Thin-layer chromatography, however, demonstrated that the predominant product with each acceptor, except for monoolein, was triglyceride. This result is incompatible with a simple lipase-catalyzed exchange reaction.

TABLE 1 COFACTOR REQUIREMENTS FOR INCORPORATION OF PALMITATE-1-C$^{14}$ INTO GLYCERIDES

| Addition or Omission | Palmitate Incorporation |
|---|---|
| | % |
| None* | 100 |
| − ATP | 2.7 |
| − CoA | 3.2 |
| − DL-α-Glycerophosphate | 4.1 |
| − DL-α-Glycerophosphate + β-glycerophosphate, 2.5 mM | 13.6 |
| DL-α-Glycerophosphate, 0.2 mM | 11.1 |
| " 1 mM | 72.1 |
| " 2.5 mM | 100 |
| − $MgCl_2$ | 16.9 |
| $MgCl_2$, 0.2 mM | 68.4 |
| $MgCl_2$, 3 mM | 124.3 |
| − NaF | 158 |
| − Phosphocreatine | 131.7 |
| + Cysteine, 3 mM | 101.9 |

* The complete system was as described in Materials and Methods with α-glycerophosphate, 10 mM, as acceptor and 0.05 ml of a 1:5 homogenate in a total volume of 2 ml. In the complete system 177,000 cpm were incorporated into glycerides. This was equivalent to approximately 50 mμmoles of palmitate.

TABLE 2 ACCEPTORS OF PALMITATE-1-C$^{14}$ IN GLYCERIDE BIOSYNTHESIS BY THE PARTICULATE FRACTION OF LACTATING MAMMARY GLAND

| Acceptor | Palmitate Incorporation | Product | |
|---|---|---|---|
| | | Triglyceride | Diglyceride |
| | *mμmoles* | % | % |
| None | 1 | — | — |
| 1,2-Diglyceride, 30 μmole | 9 | 66.8 | 24.0 |
| 1,3-Diglyceride, 30 μmole | 2 | 44.7 | 30.1 |
| Triglyceride, 20 μmole | 10 | 91.1 | 5.9 |
| Monoolein, 20 μmole | 4 | 37.2 | 60.0 |
| Phosphatidic acid, 0.6 μmole | 20 | 65.4 | 31.0 |
| DL-α-Glycerophosphate, 1.0 μmole | 49 | 55.4 | 42.0 |

### *Effect of Tween and Cofactors on Glyceride Biosynthesis*

Further evidence that incorporation of palmitate-1-C$^{14}$ was due to formation of new ester bonds rather than to exchange reactions, even in the case of the triglyceride acceptor, is shown in Table 3. The esterification of palmitate-1-C$^{14}$ was studied with the acceptors emulsified by sonication with and without Tween 80. In addition, incubations were performed in the presence and absence of ATP and CoA. In all cases, both with and without Tween, there was essentially no incorporation in the absence of ATP and CoA. These results eliminate the possibility that esterification of palmitate was simply

TABLE 3 EFFECT OF TWEEN AND COFACTORS ON GLYCERIDE BIOSYNTHESIS

| Acceptor | Palmitate Incorporation | |
|---|---|---|
| | − Tween | + Tween (1.25 mg) |
| | *mμmoles* | |
| None | 4 | 1 |
| − ATP and CoA | 0.5 | 0.1 |
| Triolein | 24 | 1 |
| − ATP and CoA | 0.5 | 0.2 |
| 1,2-Diolein | 32 | 5 |
| − ATP and CoA | 1 | 0.5 |
| 1,3-Diolein | 20 | 1.5 |
| − ATP and CoA | 1 | 0.2 |
| Monoolein | 7 | 6 |
| − ATP and CoA | 2 | 0.5 |

The concentration of all acceptors was 7.5 × $10^{-6}$ M in incubations without Tween and 2.5 × $10^{-6}$ M in incubations with Tween.

an exchange reaction catalyzed by a lipase for, even with triglyceride as acceptor, ATP and CoA were essential.

With all acceptors except monoolein, esterification of palmitate was greatly inhibited by Tween 80 (Table 3). Incorporation in the presence of triglyceride and 1,3-diglyceride was virtually eliminated by Tween 80, and incorporation due to 1,2-diglyceride was reduced by 80%. In contrast to this, esterification of palmitate with monoolein as acceptor was just as great in the presence of Tween 80 as in its absence.

Furthermore, the fact that in the presence of Tween monoolein was a better acceptor than an equimolar amount of diglyceride eliminates the possibility that the activity of the monoglyceride was due to contaminating diglyceride.

Analysis of the products formed with monoolein as acceptor in the presence of Tween 80 revealed that 90% of the radioactivity was in diglyceride. There can be no doubt, therefore, that lactating mammary gland homogenate is capable of acylating monoglycerides. This reaction, in contrast to the acylation of 1,2-diglyceride, appears not to be inhibited by Tween 80.

### *Phosphatidic Acid Concentration*

It was found in most experiments that maximal stimulation was obtained with very low concentrations of phosphatidic acid and that inhibition appeared with increasing concentrations (Table 4).

### *Esterification of Ethanol*

In preliminary experiments, the acceptors were added to the incubation mixtures as solutions in ethanol. It was soon discovered, however, that ethanol itself stimulated incorporation of palmitate-1-$C^{14}$ into the neutral lipid fraction. The reaction was enzymatic, requiring homogenate, ATP, and CoA. In one experiment, carrier ethyl palmitate was added to the radioactive neutral lipids and the mixtures separated by gas–liquid chromatography on ethylene glycol succinate polyester. Emerging fractions were collected on anthracene and radioactivity was measured directly in a scintillation spectrometer. All of the radioactivity was recovered in ethyl palmitate.

TABLE 4 Effect of Phosphatidic Acid Concentration on Glyceride Biosynthesis

| Phosphatidic Acid | Palmitate Incorporation | Product | |
|---|---|---|---|
| | | Triglyceride | Diglyceride |
| *μmoles* | *mμmoles* | % | |
| 0 | 4 | 85 | 7 |
| 0.15 | 15 | 68 | 26 |
| 0.3 | 13 | | |
| 0.6 | 15 | | |
| 1.5 | 9 | | |
| 3.0 | 7 | | |
| 6.0 | 2 | | |
| 15.0 | 0.5 | | |
| 1.5 (– ATP and CoA) | 0.3 | | |

At 0.05 M, ethanol gave detectable stimulation of the esterification of palmitate and at 2.0 M, ethanol was more active than the maximal effect of α-glycerophosphate (obtained, however, at $^1/_{1000}$ the concentration). Ethanol was inhibitory at concentrations greater than 2.0 M.

## DISCUSSION

The results clearly indicate that biosynthesis of glycerides in lactating guinea pig mammary tissue can occur by both of the previously described pathways, one involving the acylation of α-glycerophosphate with phosphatidic acid and 1,2-diglycerides as intermediates, the other the direct acylation of monoglycerides. These results differ from those of Dils and Clark (21), who studied rat mammary gland homogenates, and those of Pynadath and Kumar (22), who used extracts of goat mammary gland. In both of those earlier studies, monoglyceride was found not to serve as an acceptor for free fatty acids in glyceride synthesis. In a preliminary study, we also reported that monoglyceride was not an acceptor for glyceride synthesis (13). Our earlier results were wrong because the monoolein was poorly emulsified and used at too low a concentration.

A plausible explanation for the ability of triglyceride to act as acceptor only when ATP and CoA are present would be that the true acceptor was diglyceride formed from the triglyceride by the action of a lipase. This same explanation might apply to the experiments with 1,3-diglyceride, which might be thought to react only after cleavage to monoglyceride. Some indirect support for this hypothesis can be drawn from the data in Table 3, wherein Tween 80 inhibited esterification of palmitate much more when triglyceride and 1,3-diglycerides were the acceptors than when 1,2-diglyceride was the acceptor. This would be reasonable if the postulated lipase were inhibited by Tween 80.

An attempt to demonstrate the presence of a lipase by the production of free fatty acid from triglyceride was negative, but with that homogenate, triglyceride did not function as acceptor. The question remains, therefore, unresolved.

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### References

1. Smith, S. W., S. B. Weiss, and E. P. Kennedy. *J. Biol. Chem.* **228:** 915, 1957.
2. Stein, Y., and B. Shapiro. *Biochim. Biophys. Acta* **24:** 197, 1957.

3. Steinberg, D., M. Vaughan, and S. Margolis. *J. Biol. Chem.* **236:** 1631, 1961.
4. Clark, B., and G. Hübscher. *Biochim. Biophys. Acta* **46:** 479, 1961.
5. Senior, J. R., and K. J. Isselbacher. *Biochem. Biophys. Res. Commun.* **6:** 274, 1961.
6. Johnston, J. M., and J. L. Brown. *Biochim. Biophys. Acta*, **59:** 500, 1962.
7. Clark, B., and G. Hübscher. *Biochim. Biophys. Acta* **70:** 43, 1963.
8. Hübscher, G. *Biochim. Biophys. Acta* **52:** 582, 1961.
9. Stein, Y., O. Stein, and B. Shapiro. *Biochim. Biophys. Acta* **70: 33**, 1963.
10. McBride, O. W., and E. D. Korn. *J. Lipid Res.* **4:** 17, 1963.
11. McBride, O. W., and E. D. Korn. *J. Lipid Res.*, **5:** 442, 1964.
12. McBride, O. W., and E. D. Korn. *J. Lipid Res.*, **5:** 453, 1964.
13. McBride, O. W., and E. D. Korn. *Federation Proc.* **22:** 303, 1963.
14. Karmen, A., I. McCaffrey, and R. L. Bowman. *J. Lipid Res.* **3:** 372, 1962.
15. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233:** 311, 1958.
16. Mattson, F. H., and R. A. Volpenhein. *J. Lipid Res.* **2:** 58, 1961.
17. Kates, M. *Canad. J. Biochem. Physiol.*, **33:** 575, 1955.
18. Mangold, H. K. *J. Am. Oil Chemist's Soc.* **38:** 708, 1961.
19. Skidmore, W. D., and C. Entenman. *J. Lipid Res.* **3:** 471, 1962.
20. Dole, V. P., and H. Meinertz. *J. Biol. Chem.* **235:** 2595, 1960.
21. Dils, R., and B. Clark. *Biochem. J.* **84:** 19P, 1962.
22. Pynadath, T. I., and S. Kumar. *Life Sci.* **8:** 594, 1963.