Enzymatic Acyl Exchange to Vary Saturation in Di- and Triglycerides[1]

R.W. STEVENSON[2], F.E. LUDDY, and **H.L. ROTHBART,** Eastern Regional Research Center, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118

ABSTRACT

The reaction *rac*-glyceryl-1-palmitate-2,3-dioleate (*POO*) and palmitic acid (*P*) (excess) → glyceryl-2-oleate-1,3-dipalmitate (*POP*) + *O* catalyzed by porcine pancreatic lipase is a model for the general reaction $AAA' + A' \rightarrow A'AA' + A$. (*P*, *O*, *A* = palmitoyl, oleyl, acyl groups or palmitic, oleic, or fatty acids; *AAA'*, etc., are triglycerides). The re-esterification is accompanied by formation of palmitate-enriched diglyceride – *rac*-glyceryl-1-palmitate-2-oleate (*PO*-OH) exceeds *rac*-glyceryl-1,2-dioleate (*OO*-OH) – and glyceryl-2-monoleate (HO-*O*-OH). Buffer pH optimum for maximum *POO* conversion and palmitate enrichment in 15 min is between 6.0 and 6.5 at 38 C. Hexane is used to dissolve *P* in the oil phase. Increasing the amount of dissolved *P* by increasing the amount of hexane added increases palmitate enrichment and decreases reaction rate. At 36 C and 6.0 buffer pH (26 volume % *POO* and 15 volume % *P* in oil phase), yields after 3 hr were *POP*, 22%; *PO*-OH, 27%; HO-*O*-OH, 11%; *POO*, 27%; glyceryl-1,2,3-trioleate (*OOO*), 3%; and *OO*-OH, 10%.

INTRODUCTION

The physical properties of naturally occurring triglycerides depend not only on the molecular weight and saturation or unsaturation of the attached acyl groups, but also on the position of attachment of these groups. For example, the thermophysical properties of cocoa butter result from having largely palmitic and stearic acids in the primary position and largely oleic acid at the secondary position. Thus, to convert a more unsaturated triglyceride to a more saturated triglyceride with cocoa butter-like properties, hydrogenation and/or interesterification are inadequate. Both occur more or less at random and, in hydrogenation, some of the *cis* unsaturation is converted to *trans*.

A method is therefore needed which will provide triglycerides with the attached acyl groups in the appropriate positions for the desired physical properties. Mattson, Volpenhein, and Martin have described the esterification without rearrangement of 1,3 and 1,2 diglycerides by acid anhydride (1), and Mattson and Volpenhein have described the acid chloride esterification also without rearrangement (2). The first two authors also reviewed the synthesis and properties of glycerides (3). These methods require conversion of all the reactant acid to chloride or anhydride and elevated temperature.

Of greater advantage would be a reaction in which at least a portion of triglyceride could be converted directly to new, desired triglycerides, by use of carboxylic acid instead of the chloride or anhydride. It is well known that pancreatic lipase E.C. 3.1.1.3 catalyzes the hydrolysis of triglycerides in the primary position (4). Brockerhoff and Jensen's monograph contains many references to means for driving the reaction to the right, that is, the avoidance of the reverse reaction in which triglycerides are synthesized (5). The occurrence of this reverse reaction in lipolytic hydrolysis stimulated our research to employ lipase catalysis as a means for triglyceride resynthesis. An enzymatic process for triglyceride resynthesis would have the advantages of using the carboxylic acid directly and of operating at temperatures lower than those necessitated by other means. Okumura et al. have reported resynthesis of esters in their work with four microbial lipases (6,7). Their work appears to be directed primarily at elucidating the mode of action of microbial lipases rather than to a synthetic method of potential large scale use.

In our research, we worked on a small scale with a buffer-dissolved enzyme. The model compound *rac*-glyceryl-1-palmitate-2,3-dioleate (*POO*) was reacted with palmitic acid (*P*) with the goal of synthesizing glyceryl-2-oleate-1,3-dipalmitate (*POP*). *POP* is similar to Luddy's beef tallow semisolid fraction (8), which is in turn similar to cocoa butter (9).

MATERIALS AND METHODS

Materials

Absolute methanol, ACS grade silver nitrate, and anhydrous ether were obtained from the J.T. Baker Chemical Company (Phillipsburg, NJ). The ether was distilled before

[1]Presented in part at American Chemical Society Meetings, New Orleans and Chicago, 1977.

[2]Cheyney State College, Cheyney, Pennsylvania 19319.

TABLE I

Results of 15 Minute Reactions of *P* with *POO* at 38 C in the Presence of Varying Amounts of Hexane[a]

	Mole % of Glycerides Recovered					
μl Hexane	HO-*O*-OH	*OO*-OH	*PO*-OH	*OOO*	*POO*	*POP*
0 (No *P*)	31	23	23	7	11	5
0	28	23	23	5	17	4
25	22	15	21	6	31	6
50	14	14	24	5	35	8
75	8	12	25	2	35	18
100	10	8	24	1	42	14
125	11	12	25	2	50	7
150	4	3	10	<1	80	3

[a]36 mg *P*, 56 mg *POO*, 0.5 mg lipase, 1 ml 5.0 pH buffer, 1 min. shake on Wig-L-Bug each before and after 15 min. at 38 C.

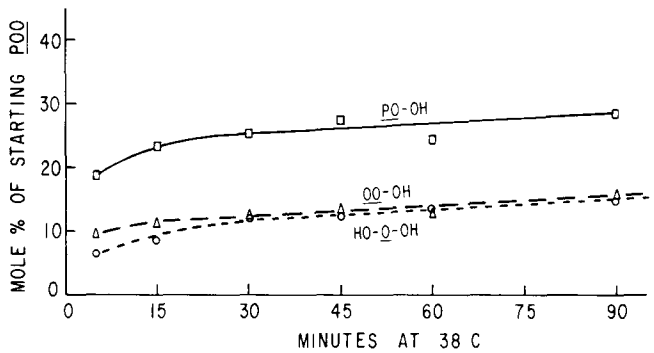


FIG. 1. Yield: Mono and diglycerides vs. time at 38 C. Yields calculated as (individual glyceride recovered x 100) ÷ (total of all glycerides recovered). Reaction of ca. 56 mg *POO* diluted with 75 μl hexane with 0.5 mg lipase in the presence of ca. 36 mg added *P* and 1.0 ml 5.0 pH buffer. Reactions run for indicated time in 38 C bath after initial 1 min shake on amalgamator.

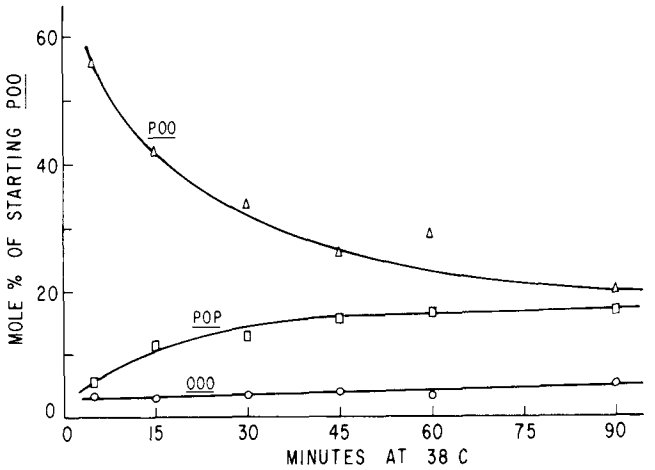


FIG. 2. Yield: Triglycerides vs. time at 38 C. Same reactions described for Fig. 1.

use. Nanograde Petroleum ether and analytical reagent grade chloroform were obtained from Mallinckrodt (St. Louis, MO). *rac*-Glyceryl-1-palmitate-2,3-dioleate (*POO*) was synthesized by J. Sampugna and R. Jensen at the University of Connecticut (10). Lipase, Type VI, (E.C. 3.1.1.3) lyophilized powder from hog pancreas was purchased from Sigma Chemical Company (St. Louis). Its reported activity was 41400 units/mg of solid, one unit of which hydrolyzes 1.0 microequivalent of acid from olive oil in 1 hr. Formic acid, practical grade, was obtained from Eastman Kodak Company, (Rochester, NY), and palmitic acid (containing ca. 1.4% stearic acid) was prepared from cottonseed oil fatty acids by solvent (acetone) fractional crystallization followed by high vacuum fractional distillation.

Silica gel G was obtained from E. Merck AG, (Darmstadt, Germany), and prepared silica gel G plates (20 x 20 cm, 250 microns thickness) were obtained from Analtech, Inc. (Newark, DE).

Buffers from pH 5.0 to 7.5 were made from NaH_2PO_4, Na_2HPO_4, and/or Na_3PO_4, ACS reagent grade, obtained from J.T. Baker Company. The pH 8.0 buffer was made from sodium borate from the same source.

Methods

Reactions were carried out in 5 ml screw cap vials in an adaptation of the analytical procedures of Luddy and coworkers (11). The vials were charged, typically, with ca. 56 mg *POO* (ca. 65 μ mole, 63 μl), 36 mg of *P*, 75 μl hexane, 0.50 mg pancreatic lipase, and 1.0 ml buffer. The vial was shaken for 1 min on the dental amalgamator (Wig-L-Bug, Crescent Dental Mfg. Company, Chicago, IL) at ambient temperature, then kept at 38 C or other desired temperature for the required time. Following some of the reactions, there was another minute shake on the amalgamator. The reaction mixture was then worked up immediately, essentially as described by Luddy et al. (11).

Class separation of the mixture was accomplished by thin layer chromatography (TLC) on silica gel G plates. The mixture was dissolved in ca. 1 ml of ether in a vial, and an aliquot of 500 μl was removed and transferred to another vial. This aliquot, typically containing 40 mg or more of acids and glycerides, was deposited across ca. 16 cm of a 20 x 20 cm plate. Development was in 80% petroleum ether, 20% ethyl ether, plus 0.4% HCOOH over 10-14 cm. After visualization with iodine vapor, bands for triglyceride, diglyceride, and monoglyceride were carefully scraped from the TLC plate, extracted with ether, and the glycerides were isolated and weighed. The extraction was carried out by transferring the silica gel containing the glyceride from the paper onto which it had been scraped into a 5 ml vial, adding ether, sealing the vial, shaking on the amalgamator, and centrifuging. The supernatant was decanted into another vial, evaporated, and weighed. Although two extractions gave good results, smoother plots of responses vs. reaction variables were obtained when three extractions were employed. Monoglyceride extraction was aided by using ca. 1 ml of absolute alcohol with ether in the first extraction of that fraction. On the assumption that there was minimal glycerol formation, the acid fraction was not usually worked up. When worked up to confirm material balance, four extractions were used for the acid fraction because of the larger volume of silica gel.

Glycerides were converted to glycerol and fatty acid methyl esters, the latter of which were analyzed by GLC (12). In calibration, concentration of the methyl ester solution by evaporation was shown not to alter the methyl oleate/methyl palmitate ratios. Diglyceride percentages – *rac*-glyceryl-1-palmitate-2-oleate (*PO*-OH), and *rac*-glyceryl-1,2-dioleate (*OO*-OH) were calculated from the oleate/palmitate ratios. Monoglyceride was analyzed to confirm its expected composition as monooleate.

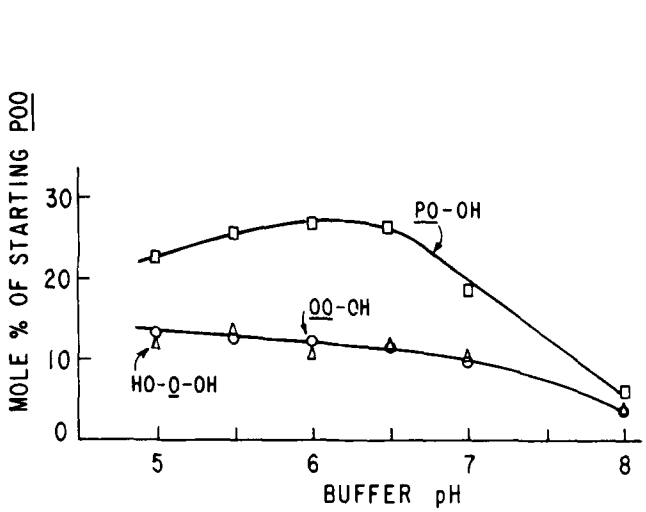
Triglycerides were analyzed by argentometric TLC. The

TABLE II

Results of 15 Minute Reactions of Hexane-Dissolved *P* with *POO* at Varying Temperatures[a]

Temperature C	HO-*O*-OH	*OO*-OH	*PO*-OH	*OOO*	*POO*	*POP*
34.4	10	13	26	N.D.	43	8
37.3	10	11	24	1	41	12
38.5	13	13	22	3	39	10
39.4	15	12	25	6	31	11
40.5	14	13	25	7	30	11
42.1	8	11	26	2	43	10

[a]Conditions same as for Table I, except that 75 μl hexane is used in each reaction.



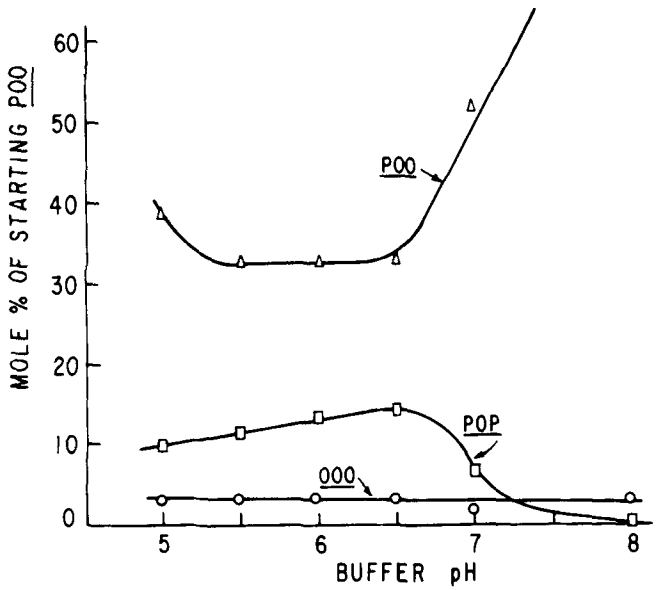


FIG. 3 & 4. Yields of mono-, di- and triglycerides as mole % of starting *POO* vs. buffer pH at 38 C. Reactions as for Figs. 1 and 2 except that buffer pH was varied from 5.0-8.0, and time was constant at 15 min. Yields calculated as for Figs. 1 and 2.

plates were prepared as follows: with lab lights off, 7 g of silica gel G was stirred into 16 ml of $AgNO_3$ solution (12.5 g $AgNO_3$/ml) for 75 sec and spread on a 20 x 20 cm glass plate at 250 micons thickness. After 2 hr at 105 C, the plates were stored in the dark until used.

Up to 15 mg of triglyceride was spotted, and the plate was developed first in 98% $CHCl_3$/2% methanol, and then in $CHCl_3$, as reported by Sampugna and Jensen (13). Separation of *POP* (monounsaturated), *POO* (diunsaturated) and *OOO* (triunsaturated) was visualized under ultraviolet light after spraying with dichlorofluorescein solution. The individual triglycerides were isolated by scraping and extracting the silica gel, as described for class separation. The composition of the fractions was established by GLC of methyl esters in a few reactions.

The analysis allows calculation of *POP*, *POO*, *PO*-OH, *OO*-OH, and glyceryl-2-monooleate (HO-*O*-OH) as mole percent of starting *POO*.

Further analysis of *POO* and diglyceride fractions was based on the fact that starting *POO* contained no stearic acid (*S*) as impurity, while the *P* employed contained ca. 1.4% stearic. The assessment by GLC of *S*/*P* ratios in material regarded as primarily *PO*-OH helped to establish the extent of the reaction.

RESULTS AND DISCUSSION

The lipase-catalyzed reaction directed to formation of glyceryl-2-oleate-1,3-dipalmitate (*POP*) and *rac*-glyceryl-1-palmitate-2-oleate (*PO*-OH) from *rac*-glyceryl-1-palmitate-2,3-dioleate (*POO*) was studied as a function of oil phase dilution (hexane), reaction time, temperature, buffer pH, and presence or absence of excess palmitic acid. Dissolved enzyme was used. The results are consistent with a reaction controlled by diffusion of the substrate in the oil phase to the reaction zone, followed by equilibration of the glycerides produced.

Dilution of Oil Phase

The use of hexane to dissolve more palmitic acid (*P*) in the oil phase was systematically studied over a range of volumes varying from 0 to 150 μl (Table I). The addition of *P* to undiluted *POO* in a lypolysis reaction gave results only slightly different from a reaction in which it was absent. Increased hexane volume would be expected to dissolve more *P* and produce larger amounts of *P*-containing products. *POP* yields of 14-18% are obtained at 100-75 μl hexane. *PO*-OH changes little between 0 and 100 μl, while *rac*-glyceryl-1,2-dioleate (*OO*-OH) and glyceryl-2-monooleate (HO-*O*-OH) decrease throughout as hexane volume increases.

The reaction is so slow at 125 and 150 μl added hexane that it progresses relatively little in 15 min. Since the initial reaction is probably controlled by diffusion of *POO* to the reaction site, there should be an appreciable amount of *POO* that has never had an opportunity to react at the higher dilutions. Palmitate incorporation then reflects not only the relative amounts of free palmitic and oleic acids available, but also the availability of *POO* to the reaction site.

Reaction Time

Using 75 μl added hexane in reactions, we studied reaction time after initial 1 min shaking for from 5 to 90 min. Glyceride yields are shown in Figures 1 and 2. Although the slope of the curve at 90 min is low, careful analysis at long reaction times demonstrates that product yield is still increasing, showing that the enzyme maintains activity over this period. However, some activity may be lost due to irreversible unfolding of the enzyme at the hydrocarbon and oil/water interface (14). It is also possible that the reaction is slowed by larger oil drops in the emulsion during the later stages of reaction, so that *POO* takes longer to diffuse. Borgström, who was the first to demonstrate reversbility of the hydrolysis of glycerol-1,2,3-trioleate in the presence of radioactively labeled oleic acid, stated that resynthesis is favored by coarse emulsions (15); thus, we did not attempt to maintain fine emulsions.

At longer times, nearly constant levels of products (as % of *POO* reacted) have been achieved, indicating that most of the *POO* may have had an opportunity to diffuse into the reaction zone.

Temperature

We studied reaction temperature between 34 and 42 C. Higher temperatures would be expected to favor greater solubility of palmitic acid in the oil phase, hence greater palmitate incorporation in resynthesized di- and triglycerides. However, higher temperatures in this range would be expected to accelerate inactivation of the enzyme.

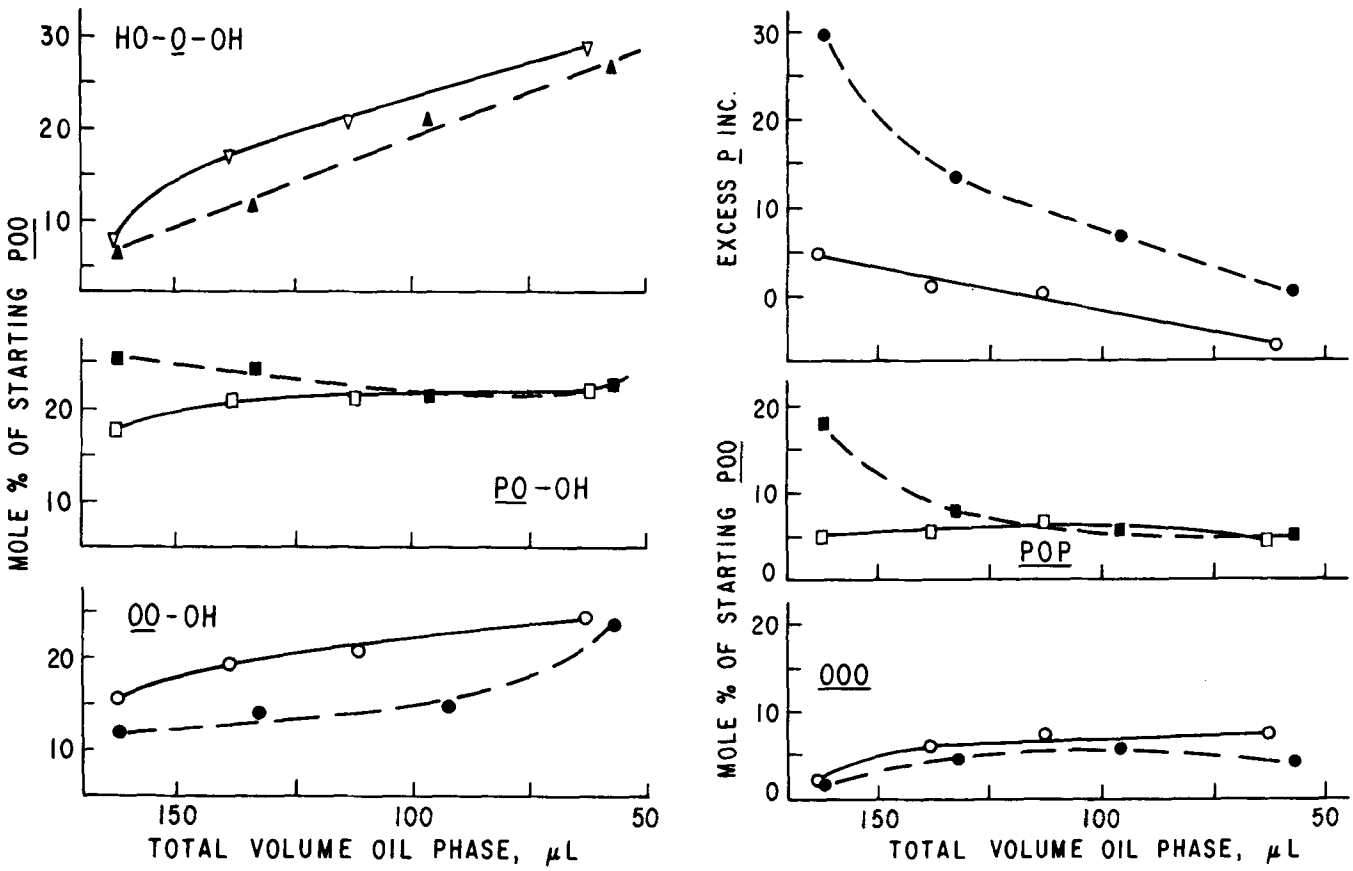


FIG. 5 & 6. Yields of mono-, di- and triglyceride vs. total volume oil phase with and without added palmitic acid. Reactions as for Figs. 3 and 4, except buffer pH was 5.0, and varying amounts of hexane were used. Filled symbols are for reactions with added *P*. Top of Figure 6: Excess *P* incorporated vs. total volume oil phase (for random hydrolysis and reesterification, *PO*-OH = *OO*-OH, and *POP* = *OOO* would be expected; *PO*-OH-*OO*-OH + *POO*-*OOO* when not zero represents nonrandom fatty acid incorporation). Dotted lines follow filled symbols; solid lines follow open symbols.

Table II shows the yields of glycerides. *PO*-OH tends to be approximately constant from 34-42 C. Glyceryl-1,2,3-trioleate (*OOO*), HO-*O*-OH and *OO*-OH increase with temperature, except at 42 C. At 42 C, the enzyme has probably been inactivated before the end of the indicated 15 min reaction time.

pH Optimum

Discussions of lipolytic hyrolysis usually indicate a pH optimum of 8.0 (16). Borgström reports a buffer pH of 5.6 to be best for resynthesis with oleate (15). Figures 3 and 4 show glyceride yields as a function of buffer from 5.0 to 8.0. A standard 15 min reaction time was used in each case. From our data, optimum buffer pH for production of *PO*-OH and *POP* is ca. 6.0 to 6.5.

Effect of Oil Phase Dilution on Palmitate Incorporation with and without Excess Palmitic Acid

Various parameters can affect the relative rates of hydrolysis and presumably resynthesis of triglycerides by lipase (5,17). The purpose of hexane addition is to increase the amount of acid in the oil phase. The question of whether the dilution of the oil phase in itself contributes to palmitate enrichment in resynthesized triglycerides is treated in Figures 5 and 6. Glyceride yields are plotted against total volume of oil phase, diminishing volume to the right. Total volumes were approximated by assuming ideality. Curves for *PO*-OH and *POP* with and without palmitic acid converge with decreasing oil phase volume. As the oil phase volume is increased by addition of hexane, the effect of added palmitic acid is to produce more *PO*-OH and *POP*. Less HO-*O*-OH, *OO*-OH, and *OOO* are produced throughout the entire range when palmitic acid is present. Excess palmitate produced by the reactions is plotted in Figure 6 to show palmitate incorporation increasing with volume of oil phase, both in the presence and absence of added palmitic acid. At each oil phase volume, considerably more palmitate incorporation is obtained in the presence of added palmitic acid. In the absence of added palmitic acid, palmitate incorporation is negative for low dilutions (more oleic acid reincorporated than palmitic). This may be attributed to the limited solubility of palmitic acid in the oil phase in the undiluted system, so that perhaps even the small amount of palmitic acid from the hydrolysis has exceeded its solubility limit.

Increasing the Yield of *POP* and *PO*-OH

Palmitate incorporation is best at buffer pHs of 6.0 and 6.5. Also, increasing hexane dilution of the oil phase dissolves more *P* and increases the yield of palmitate-containing products. Since this dilution also slows the reaction, less *POO* is converted in a given time and longer reaction time is required to compensate. Table III shows results of reactions carried out at 36 C and 6.0 pH. The lower temperature was used to lessen enzyme inactivation during longer reactions, and also, selectivity for palmitate over oleate seemed better at 36-37 C. Runs of 60 min or more appear to be near completion with respect to *POP* synthesis.

The Triglyceride Reaction

Under most conditions, the formation of *POP* appears to depend upon the concentration of *P* in solution in the oil phase. *P* in solution relative to *O* also appears to control the

TABLE III

Time and Hexane Dilution of Oil Phase 6.0 pH and 36 C

Min.	μl Hexane	Mole % of starting *POO*					
		HO-*O*-OH	*OO*-OH	*PO*-OH	*OOO*	*POP*	*POO*
15	100	12	12	23	3	13	36
15	125	10	9	23	2	14	42
90	125	14	13	28	6	19	20
180	125	12	12	27	6	20	22
60	150	9	9	24	3	18	37
180	150	11	10	27	3	22	27

PO-OH/*OO*-OH ratio, probably through esterification of 2-monoolein which appears early in the reaction. Observed yields of resynthesized triglycerides are probably lower than attainable yields, since the reaction is controlled by diffusion of *POO* to the reaction site (18). At 36 C, 150 μl hexane, 6.0 buffer pH, and 180 min, observed yields (Table III) are *POP*, 22%; *OOO*, 3%; *PO*-OH, 27%; *OO*-OH, 10%, HO-*O*-OH, 11%.

Monoglyceride and Diglyceride Reactions

Monoglycerides and diglycerides appear to form more rapidly than resynthesized triglycerides. *PO*-OH/*OO*-OH ratios correspond to the estimated *P/O* ratios in solution in the oil phase early in the reaction, while resynthesized triglycerides correspond to this ratio only after longer reaction times.

PO-OH from the lipolysis of *POO* in the presence of excess palmitic acid can come either from hydrolysis of *POO* or from the reesterification of HO-*O*-OH with *P*. We did not use radiotracers in our work, but there was stearic acid (*S*) in the excess *P* employed ($S/P = 1.4 \times 10^{-2}$), and no detectable *S* in the *POO* with which we started.

Using the estimate of how much of the starting *POO* has reacted, we can predict the *S/P* ratio expected in *PO*-OH completely equilibrated with *P* in solution. The *P* in solution represents *P* hydrolyzed from *POO* (containing no *S*) mixed with added *P* (containing *S*). *S/P* ratios calculated for *P* in solution therefore will be less than 1.4×10^{-2}. The dilution of the *P* containing *S* with pure *P* (from hydrolysis) is calculated from stoichiometry. For complete equilibration, the average *S/P* in *PO*-OH for 14 successive runs (Table I, plus two other runs) was predicted to be 1.10×10^{-2} and found to be 1.13×10^{-2}, in excellent agreement. (Individual values ranged from 0.98 to 1.30×10^{-2} for calculared, 0.92 to 1.33×10^{-2} for found.) This result is consistent with the rapid equilibration of diglyceride with dissolved acids in the oil phase. Further discussion of the nature of the reaction will be published shortly.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Fats and Proteins Research Foundation to R.W. Stevenson. The latter is also grateful to Cheyney State College for Sabbatical Leave during the 1976-1977 academic year. R.A. Barfod, P. Magidman, P.E. Pfeffer, and J. Sampugna provided assistance, advice, and instruction.

REFERENCES

1. Mattson, F.H., R.A. Volpenhein, and J.B. Martin, J. Lipid Res. 5:374 (1964).
2. Mattson, F.H., and R.A. Volpemhein, Ibid. 2:58 (1961).
3. Mattson, F.H., and R.A. Volpenhein, Ibid. 3:281 (1962).
4. Mattson, F.H., and L.W. Beck, J. Biol. Chem. 214:115 (1955); 219:735 (1956).
5. Brockerhoff, H., and R.G. Jensen, "Lipolytic Enzymes," Academic Press, New York, 1974, p. 34.
6. Okumura, S., M. Iwai, and Y. Tsujisaka, Agric. Biol. Chem. (Jpn.) 40:655 (1976).
7. Tsujisaka, Y., S. Okumura, and M. Iwai, Biochim. Biophys. Acta 489:415 (1977).
8. Luddy, F.E., J.W. Hampson, S.F. Herb, and H.L. Rothbart, JAOCS 50:240 (1973).
9. Hampson, J.W., F.E. Luddy, and H.L. Rothbart, Candy and Snack Industry 140, No. 11:37.
10. Jensen, R.G., T.A. Marks, J. Sampugna, J.G. Quinn, and D.L. Carpenter, Lipids 1:451 (1966); J.G. Quinn, J. Sampugna, and R.G. Jensen, JAOCS 44:439 (1967).
11. Luddy, F.E., R.A. Barford, S.F. Herb, P. Magidman, and R.W. Riemenschneider, Ibid. 41:693 (1964).
12. Luddy, F.E., R.A. Barford, S.F. Herb, and P. Magidman, Ibid. 45:549 (1968).
13. Sampugna, J., and R.G. Jensen, Lipids 4:444 (1969).
14. Brockerhoff, H., J. Biol. Chem. 246:5828 (1971).
15. Borgström, B., J. Lipid Res. 5:522 (1964).
16. Mattson, F.H., and R.A. Volpenhein, JAOCS 43:286 (1966).
17. Desnuelle, P., in "The Enzymes," Vol. III, Third Ed., Edited by P.D. Boyer, Academic Press, New York, 1972, p. 575.
18. Desnuelle, P., M.J. Constantin, and J. Baldy, Bull. Soc. Chim. Biol. 37:285 (1955).

[Received February 7, 1979]