Lipase-Mediated Acyl-Exchange Reactions with Butteroil in Anhydrous Media

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Conditions optimum for porcine pancreatic lipase catalyzed acyl-exchange reactions between a free fatty acid (FFA), undecanoic acid, and butteroil in anhydrous media were established. No solvent was required for reaction, indicating that butteroil could act as dispersant as well as substrate in reactive mixtures. Optimum temperature and pH for the reaction were 70°C and 6.5 to 7.0, respectively. The addition of up to 550 mM water to reactive mixtures had little influence on the initial rates of acyl-exchange, but shifted the reaction equilibrium to favor net hydrolysis. Optimal FFA concentration for acyl-exchange was 250 mM in terms of initial reaction rates, and substrate inhibition by FFA was apparent at levels up to 1000 mM. In terms of % reaction yield and absolute reaction yield after 18 hr, 50 mM and 250 mM FFA, respectively, were optimum. Initial reaction rates for acyl-exchange between two model triacylglycerides indicated that esterified fatty acids were better substrates than FFA under the conditions evaluated.

KEY WORDS: Acyl-exchange, anhydrous, butteroil, lipase, modification.

Three means to tailor lipids for specific applications in food systems are genetic manipulation of lipid biosynthesis in living organisms (1), environmental control of lipid biosynthesis during fermentation of microorganisms (2), and the use of enzymes to selectively modify food lipids (3). Although the use of lipases to modify food lipids through acyl-exchange reactions is commonplace (3), other enzymic modifications, particularly at mid-chain sites on fatty acyl residues, have been identified (4).

There has been much recent interest in the study of enzyme action in nonaqueous media. Many enzymes are active in organic solvents, with the extent of activity being partially dependent on the choice of solvent (5,6). Solvents conducive to promoting enzyme activity in the absence of water are those with a limited affinity for water, such as hexane, isooctane, dodecane, ethyl ether and butyl acetate. Water-miscible solvents, such as acetone, acetonitrile, tetrahydrofuran, dimethysulfoxide and formamide tend to strip conformational water from the enzyme and prevent full expression of activity.

The use of nonaqueous media has the advantage of making conditions more favorable for enzyme action on apolar substrates compared to aqueous systems. In addition, the dilution of enzyme by water is avoided. In the special case of interfacial enzyme reactions, the use of hydrophobic organic solvents favors a one-phase reaction system which may be more efficient than the typical two-phase system (7). Enzymes also are more thermostable in nonaqueous than in aqueous media (5,8–10), and enhanced thermostability of enzymes is an area targeted for future development (11). For hydrolytic enzyme reactions, the deprivation of water also favors synthetic reactions. This has been shown for lipase-mediated esterification and acyl-exchange reactions on lipids in nonaqueous media at low water activities (3,12,13). Generally, organic solvents are used as dispersing media for lipase (3,12) and other enzyme (6) reactions at low water activities. However, two recent reports have shown that lipase-catalyzed acyl-exchange (14) and esterification (15) reactions with acylglycerides (AGs) do not always require the presence of an organic solvent. Unfortunately, these studies provided little detail on the effect of environmental parameters on the rate of these enzymic processes.

The intent of this work was to evaluate and optimize the environmental factors governing acyl-exchange reactions between butteroil and fatty acyl substrates using porcine pancreatic lipase in nonaqueous media. Butteroil ranks third behind soybean and palm oils in global production (1) and is a food lipid expected to be in increasing surplus as consumers attempt to limit their intake of saturated fat and cholesterol.

EXPERIMENTAL

Materials. Anhydrous butteroil, specified by the manufacturer to be ≤ 0.15% water, was obtained from Level Valley Dairy (West Bend, WI) and stored at 2–4°C. Prior to use, the butteroil was melted at 50°C and combined with sodium sulfate (10%, w/w) for 30 min to remove any residual water. Triacylglyceride (TAG) and free fatty acid (FFA) standards, *p*-bromophenacyl bromide, 1,4,7,10,13,16-hexaoxacyclooctadecane, lecithin and crude porcine pancreatic lipase (37% protein, 53 U/mg solid) were obtained from Sigma Chemical Co., St. Louis, MO. The lipase was held under vacuum in a desiccator at 22°C for at least 24 hr prior to use. The moisture content of the enzyme powder was measured with a Karl Fischer titrimeter (Model 691, Fisher Scientific Co., Fairlawn, NJ). All solvents used were high-performance liquid chromatography (HPLC) grade and were obtained from Baker Chemical Co., Phillipsburg, NJ. All other chemicals were reagent grade or better (VWR Scientific, Chicago, IL).

Reaction conditions. Mixtures of 10 mL, consisting of various combinations of butteroil, hexane, FFA and TAG, were placed in a tightly capped, Teflon-lined 15 mL vial. The mixture was preheated to 60°C (unless otherwise indicated) in a water-jacketed vessel, and the contents of each vial subjected to magnetic stirring (900 rpm) for the duration of the experiment. Once the

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desired temperature was reached, the reaction was initiated by the addition of 1 g of enzyme powder. At selected intervals, samples (ca. 0.5 g) were removed from the reaction mixture, and reactivity was quenched by the addition of 5 mL chloroform:methanol (1:1, v/v), containing heptadecanoate (1 g/L) and/or tritridecanoin (1 g/L) as internal standard(s). Quenching of reactivity was verified by the observation that no further changes in esterified fatty acid (EFA) content or FFA content took place for up to 5 days after sampling. Reaction velocities were linear over the initial 60 min for all conditions evaluated. This was verified by analyzing samples taken at 0, 15, 30, 45 and 60 min in all cases.

For evaluating the effect of enzyme pH on the reaction, solutions of enzyme (0.25 g/mL) were adjusted to various pHs with 0.1 N NaOH or 0.1 N HCl, lyophilized and desiccated (as described earlier) prior to use.

Fatty acid analysis. FFAs in the reactive mixtures were analyzed as their *p*-bromophenacyl ester derivatives by a high-pressure liquid chromatography (HPLC) method using a reverse-phase column at 10°C (16). EFAs in the reactive mixtures were analyzed as their methyl ester derivatives by gas-liquid chromatography (GLC) using a GP 10% SP-2330, 100/120 mesh, Chromosorb WAW glass column (Supelco, Bellefonte, PA) (17). Quantification of fatty acids was done relative to external and internal (heptadecanoic acid or methyl heptadecanoate) standards.

Acylglyceride (AG) analysis. Chloroform:methanol extracts of quenched reaction mixtures were filtered over a 0.45 μ nylon membrane and separated by HPLC using a reverse-phase column at 24°C with a mobile phase of acetone:acetonitrile (4:1, v/v) at a flow rate of 1 mL/min. Peak detection was done by a Varex ELSD II mass detector (130°C) using nitrogen at 45 psig and at a flow rate of 45 mL/min to atomize the sample. Quantification was done relative to external and internal (trioctanoylglycerol) TAG standards.

RESULTS AND DISCUSSION

General considerations. The progress of acyl-exchange reactions was followed by analysis for the disappearance of specific FFA or by the appearance of specific EFA (or both). These analyses gave almost identical results (data not shown). Therefore, either method was judged suitable, and, typically, the disappearance of a specific FFA was used to follow the course of acyl-exchange in reactive mixtures. The FFA used in these experiments was undecanoic acid (C11:0) since it is present in butteroil at only trace levels (18). This made the course of the reaction easy to follow. Acyl-exchange in these reaction mixtures was enzymic. Mixtures void of lipase or those to which a heat denatured lipase (130°C for 1 hr) was added showed no evidence of acyl-exchange.

Although the use of 1 g enzyme powder per 10 mL of reaction mixture may appear excessive, it should be noted that the enzyme preparation used is estimated to be only 1% pure (19). The enzyme was insoluble in hexane or butteroil (or in a mixture thereof) and was essentially "immobilized" under the conditions used. Since the enzyme was merely suspended in these reactive mixtures, it could be recovered by simple filtration or low speed centrifugation. The enzyme retained nearly 100% activity after being recovered from a reactive mixture that had been incubated at 60°C for 18 hr. Thus, using the enzyme in this manner would allow repeated use.

Effect of solvent. The initial rate of acyl-exchange between free undecanoic acid (C11:0) and butteroil AGs at 60°C as influenced by the amount of hexane in the reactive mixtures is shown in Figure 1. As the amount of butteroil was increased to 5% (v/v) of the dispersing medium, the rates of C11:0 esterification to butteroil AGs were greatly accelerated. This was probably due to the mass action effect of increasing levels of the butteroil AG substrates to serve as acyl acceptor. As the butteroil content was increased further, little effect on reaction rates was observed, except for a decline in rate of C11:0 esterification at 100% butteroil.

The increase in viscosity attendant with increasing the butteroil content from 50% to 100% of the dispersing medium may be responsible for some of the decline in activity. However, the vigorous agitation of these reactive mixtures (magnetic stirring at 900 rpm) would appear to minimize any restrictions in mass transfer posed by a 100% compared to a 50% butteroil system. Alternatively, this decline in activity may be due to acyl-exchange reactions becoming more favored between EFAs of butteroil AGs than between free C11:0 and EFAs of butteroil AGs. By measuring only the rate of free C11:0 esterification to butteroil AGs, we were evaluating only one of several possible acyl-exchange reactions. Thus, one can speculate that the acyl-exchange activity of lipase in 100% butteroil may be as high as any other condition evaluated. More importantly, these data show that lipase was active in mixtures void of organic solvent, and butteroil could act as the dispersing medium as well as substrate for these reactions. A similar finding was recently reported by others (14). The advantages of not requiring an organic solvent for this reaction include the avoidance of using solvents of questionable toxicity and the abatement of costs required to separate the solvent from the

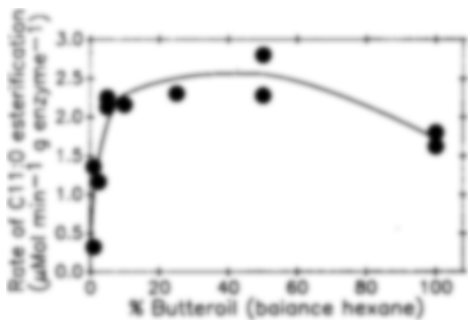

FIG. 1. The effect of presence of solvent in the dispersing phase on initial rates of acyl-exchange between free C11:0 and butteroil AGs. Butteroil (% total vol) and hexane composed the 10-mL reaction vol to which 0.50 mmoles free C11:0 and 1.0 g lipase were added. Reaction temperature was 60°C. Results are the composite of 2 experiments.

product at the termination of the reaction. Due to this observation, all further experiments were done in the absence of hexane.

Effect of temperature. The optimum temperature for initial rates of acyl-exchange reactions between free C11:0 and butteroil was 70°C (Fig. 2). In comparison, a temperature optimum of 50°C was observed for initial rates of lipase hydrolysis of a 2% (v/v) tributanoylglycerol in water emulsion (pH 7.0) containing 0.2% (w/v) lecithin and 1 mg enzyme/mL as assayed by titrimetry (data not shown). Enhanced thermostability of lipase in anhydrous media has been reported previously (8,20) and is believed to be due to the relative lack of water available to facilitate protein unfolding at high temperatures (6,8). In the present study, the use of higher levels of enzyme in the experiments in anhydrous medium compared to the aqueous medium may have contributed to the apparently higher temperature optimum observed in the former case.

The temperatures at which lipase could catalyze acyl-exchange reactions in butteroil with maximum efficiency may also be conducive to achieving pasteurization of the substrate, rendering the product free of pathogens. The activation energy (E_a) for the reaction between 40° and 70°C was calculated to be 4.0 kcal/mole. Although this may appear to be low, similar values have been calculated for the hydrolysis of butteroil by microbial lipases (1.3 kcal/mol, (21); 5.9 kcal/mole, (22)).

Effect of pH. The concept of pH is difficult to interpret in anhydrous media. However, the effect of "enzyme" pH can be evaluated and it has been proposed that enzymes (and proteins, in general) have a "memory" of the pH of the last environment to which they were exposed (6,10). With these qualifications, the effect of pH on the initial rates of acyl-exchange between free C11:0 and butteroil is shown in Figure 3. Maximum reaction rates were observed between pH 6.5 to 7.0. Activity decreased markedly as enzyme pH was increased to 8.5, suggesting the importance of the ionization state of histidine. Histidine has been reported to be a part of the catalytic triad of lipase (23). Since the pH of the enzyme reagent was about 6.0, it was judged to be of little advantage to adjust the enzyme pH prior to use. In fact, the lyophilization treatment alone reduced activity by about 37%, offsetting any gain obtained by adjusting the enzyme to an optimum pH.

Effect of added water. Typically, no water was added to the reactive mixtures, and the water content of the desiccated enzyme was 0.93 ± 0.25 (s.d) g per 100 g enzyme powder. As added water content was increased from 0 to 50 mM, an increase in the initial rate of acyl-exchange between free C11:0 and butteroil was observed (Fig. 4). The addition of low amounts of water may enhance reaction rates by increasing the rate of the initial step (hydrolysis) of acyl-exchange. This would elevate the steady-state concentration of reaction intermediates (12). At higher levels of added water, initial rates of acyl-exchange remained fairly constant.

A more pronounced influence of added water was observed in an analysis of the progress curves for FFA accumulation (not including free C11:0) in the reactive mixtures (Fig. 5). As the water content increased, so did the levels of FFA accumulating in the reactive

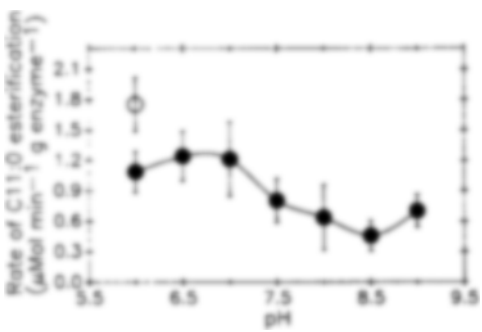

FIG. 3. The effect of "enzyme pH" on initial rates of acyl-exchange between free C11:0 and butteroil AGs. Reaction conditions were 10 mL butteroil, 0.50 mmoles free C11:0, 1.0 g lipase and 60°C. Closed symbols represent activity of enzyme reagent that had been pH-adjusted and lyophilized prior to use. Open symbol represents activity of enzyme reagent used without any modification. Results are means ± s.d. for 3 experiments.

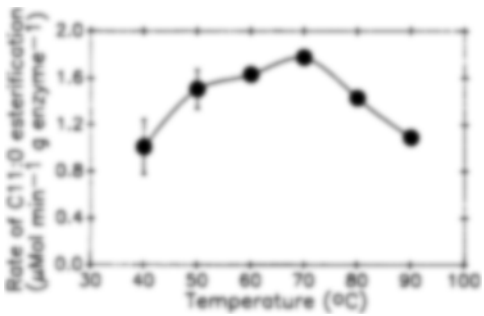

FIG. 2. The effect of temperature on initial rates of acyl-exchange between free C11:0 and butteroil AGs. Reaction conditions were 10 mL butteroil, 0.50 mmoles free C11:0 and 1.0 g lipase. Results are means ± s.d. for 3 experiments.

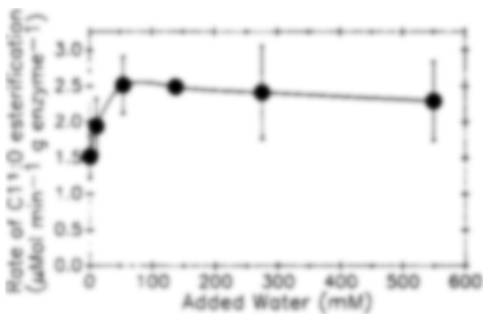

FIG. 4. The effect of added water content on initial rates of acyl-exchange between free C11:0 and butteroil AGs. Reaction conditions were 10 mL butteroil, 0.50 mmoles free C11:0, 1.0 g lipase and 60°C. Results are means ± s.d. for 3 experiments.

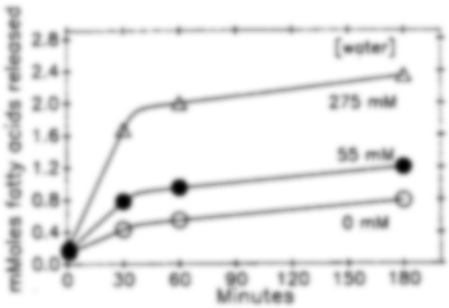

FIG. 5. Progress curves for acyl-exchange reactions between free C11:0 and butteroil AGs at 3 levels of added water. Reaction conditions were the same as in Figure 4. Results are representative of 3 experiments.

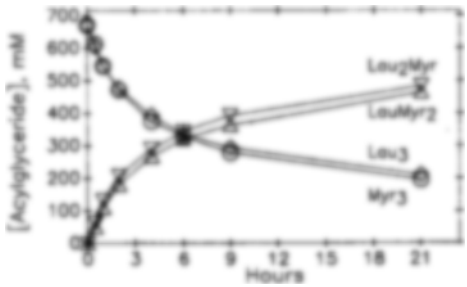

FIG. 7. Progress curves for acyl-exchange reactions between model TAGs. Reaction conditions were 660 mM each of tridodecanolyglycerol (Lau_3; ◇) and tritetradecanoylglycerol (Myr_3; ○) in 10 mL total vol, 1.0 g lipase at 60°C. Reaction products are designated didodecanoylmonotetradecanoylglycerol (Lau_2Mry; ▽) and monododecanoylditetradecanoylglycerol ($LauMyr_2$; △). Results are representative of 3 experiments.

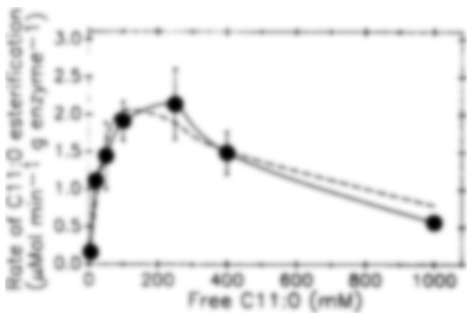

FIG. 6. The effect of FFA concentration on initial rates of acyl-exchange between free C11:0 and butteroil AGs. Reaction conditions were 10 mL butteroil, 0.050 to 10.0 mmoles free C11:0, 1.0 g lipase and 60°C. Results are means ± s.d. for 3 experiments. Dashed line represents modelled behavior of FFA inhibition by an uncompetitive mechanism (see text for details).

mixtures, and the levels of FFA accumulating were similar to the levels of water added (on a molar basis). This may be due to a shift in reaction equilibrium to favor hydrolysis (3,13). Thus, under the conditions typically used in this study, acyl-exchange reactions appeared to be most favored by the absence of any added water.

Effect of FFA concentration. As the concentration of FFA (C11:0) was increased to 250 mM, the initial rate of acyl-exchange between free C11:0 and butteroil increased (Fig. 6), probably due to a mass action effect of increased levels of FFA substrate. Above 250 mM free C11:0, activity progressively declined. These data appeared to fit a Michaelis-Menten model for simple uncompetitive inhibition by substrate—FFA—as evaluated by non-linear regression. The model used was:

$$\text{Velocity} = \frac{V_m \times S_0}{[S_0 (1 + S_0/K_i) + K_m]}$$

where S_0 is the initial FFA concentration, and the constants V_m (maximum velocity), K_i (inhibition constant) and K_m (Michaelis constant) were determined to be 5.65 μmol/min/g enzyme, 162 mM and 114 mM, respectively. One mechanism of substrate inhibition may be the partitioning of FFA at the enzyme/butteroil interface, limiting the access of other butteroil substrates to the enzyme. Fatty alcohols inhibit pancreatic lipase hydrolysis of long-chain TAGs by partitioning between the hydrophobic core and surface of the substrate (24). Alternatively, the high levels of free or ionized carboxylic acid groups may desorb some of the water from the enzyme and limit full expression of activity. A third possible explanation is that high levels of FFA may acidify the aqueous microenvironment of the enzyme resulting in a corresponding loss in activity.

Progress curves (not shown) for the reactions at different initial levels of free C11:0 indicated that the highest reaction yields of C11:0 esterification to butteroil AGs after 18 hr were observed at the lower FFA concentrations used (5 mM, 76%; 20 mM, 90%; 50 mM, 90%; 100 mM, 86%; 250 mM, 61%; 400 mM, 36%; 1000 mM, 7.5%). Maximum incorporation of free C11:0 (1.52 mmoles) into butteroil AGs after 21 hr was observed at 250 mM FFA.

Acyl-exchange between model triacylglycerides (TAGs). Due to the inhibitory effect of high levels of FFAs on acyl-exchange reactions with butteroil (Fig. 6), the reactivity of lipase with model TAGs was evaluated. A progress curve for the reaction of tridodecanoylglycerol (Lau_3; 36 acyl-carbons) and tritetradecanoylglycerol (Myr_3; 42 acyl-carbons) is shown in Figure 7. The 38 (Lau_2Myr) and 40 ($LauMyr_2$) acyl-carbon products of the reaction accumulated at the expense of the disappearance of the two TAG substrates. After 21 hr of reaction, the ratio of the 36:38:40:42 acyl-carbon TAG species was 1.1:2.5:2.4:1.0, approaching the theoretical equilibrium ratio of 1:3:3:1. The initial rate of acyl-exchange, as calculated from the disappearance of Lau_3 or Myr_3 (1980 mM acyl groups each), was about 21 μmol acyl-groups exchanged/min/g enzyme. This was about 8 times greater than the initial rates of free C11:0 esterification to butteroil AGs at 250 mM free C11:0, the optimum concentration for

activity, and about 30 times the rate observed at 1000 mM free C11:0 (Fig. 6). While some of this difference may be accounted for in the nature of the acyl-acceptor (36–42 acyl-carbon TAGs, compared to butteroil AGs), it appears that EFAs are more suitable substrates for lipase-mediated acyl-exchange reactions in anhydrous media than are FFAs. Research in progress is evaluating the acyl-group preferences of lipase-mediated acyl-exchange reaction with butteroil and model TAGs in nonaqueous media.

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REFERENCES

1. Jeffcoat, R., *Biochem. Soc. Trans. 17*:1137 (1989).
2. Shimizu, S., H. Kawashima, K. Akimoto, Y. Shinmen and H. Yamada, *J. Am. Oil Chem. Soc. 66*:342 (1989).
3. Macrae, A.R., *Biochem. Soc. Trans. 17*:1146 (1989).
4. Hammond, R.C., *Fat Sci. Technol. 90*:18 (1988).
5. Zaks, A., and A.M. Klibanov, *Proc. Natl. Acad. Sci. 82*:3192 (1985).
6. Zaks, A., and A.J. Russell, *J. Biotech. 8*:259 (1988).
7. van der Padt, A., M.J. Edema, J.J.W. Sewalt and K. van't Riet, *J. Am. Oil Chem. Soc. 67*:347 (1990).
8. Zaks, A., and A.M. Klibanov, *Science 224*:1249 (1984).
9. Ahearn, T.J., and A.M. Klibanov, *Ibid. 228*:1280 (1985).
10. Russell, A.J., and A.M. Klibanov, *J. Biol. Chem. 263*:11624 (1988).
11. Wasserman, B.P., *Food Technol. 44(4)*:118 (1990).
12. Harwood, J., *Trends Biochem. Sci. 14*:125 (1989).
13. Halling, P.J., *Fat Sci. Technol. 92*:74 (1990).
14. Kalo, P., H. Huotari and M. Antila, *Milchwissenschaft 45*:281 (1990).
15. Schuch, R., and K.D. Mukherjee, *Appl. Microbiol. Biotechnol. 30*:332 (1989).
16. Elliott, J.M., B. de Haan and K.L. Parkin, *J. Dairy Sci. 72*:2478 (1989).
17. Christie, W.W., in *Lipids Analysis*, Pergamon Press, New York, NY, 1982, pp. 51–61.
18. Christie, W.W., in *Developments in Dairy Chemistry — 2. Lipids*, edited by P.F. Fox, Applied Science Publishers, New York, NY, 1983, pp. 1–35.
19. Kirchner, G., M.P. Scollar and A.M. Klibanov, *J. Am. Chem. Soc. 107*:7072 (1985).
20. Montet, D., M. Pina, J. Grialle, G. Renard and J. Grimaud, *Fat Sci. Technol. 91*:14 (1989).
21. Kamaly, K.M., K. Takayama and E.H. Marth, *J. Dairy Sci. 73*:280 (1990).
22. Alhir, S., P. Markakis and R.C. Chandan, *J. Agric. Food Chem. 38*:598 (1990).
23. Winkler, F.K., A. D'Arcy and W. Hunziker, *Nature 343*:771 (1990).
24. Ferreia, G.C., and J.S. Patton, *J. Lipid Res. 31*:889 (1990).

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