

Interaction of beef liver lipase with mixed micelles of tripalmitin and Triton X-100

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ABSTRACT When concentrated dispersions of tripalmitin in Triton X-100 are added to reaction mixtures containing soluble beef liver lipase, the rate of hydrolysis of tripalmitin increases with incubation time. When the diluted substrate is aged at 37°C for 3 hr before the addition of enzyme, the rate of hydrolysis is greater than the rate with freshly diluted dispersions and is constant for at least 2 hr. The reciprocal of the rate of hydrolysis is a complex function of the reciprocal of the substrate concentration when measured with freshly diluted substrate dispersions. A linear relationship between these reciprocals is obtained when measured with aged preparations of substrate. The rate and extent of increase of the velocity of hydrolysis of the aged substrate in relation to the velocity of hydrolysis of freshly diluted substrate are directly proportional to the substrate concentration and inversely proportional to the Triton X-100 concentration. The apparent V_{max} of beef liver lipase for tripalmitin in diluted and aged dispersions is independent of the Triton X-100 concentration, while the apparent K_m is inversely proportional to the Triton X-100 concentration. The apparent K_m for tripalmitin complexes at zero Triton X-100 concentration was judged to be 7.5×10^{-5} M. The molecular size of dispersion complexes does not change significantly as dispersions are aged. The spherical diameter of the complexes assessed by gel filtration techniques is in the order of 100 Å.

SUPPLEMENTARY KEY WORDS kinetics · glycerol tripalmitate-1-¹⁴C

THE LIPASES of rat liver have been extensively investigated. It has been shown that rat liver contains acid (1) and alkaline (2) triglyceride lipase activity and a membrane-bound lipase with an acid pH optimum

(3). A lysosomal acid lipase has been partially purified (4), and a microsomal lipase has been shown to have an alkaline pH optimum (5). Thus, the rat liver lipase activity is the result of a group of enzymes with varied properties and localization. We have begun an investigation of the beef liver lipase activity since beef liver is a readily available source of large quantities of lipase activity from which appreciable amounts of the individual lipases can be obtained. Early in these investigations, it was found that the beef liver lipase activity is markedly sensitive to alterations in the properties of micellar dispersions of substrate.

The intracellular triglyceride in normal liver cells exists in part as micellar complexes which are stabilized by biochemical detergents. Triglycerides in this form are substrates for liver lipase. The dispersion state of liver triglycerides is altered to a liquid droplet form in diseased states such as fatty liver (6). The preparation of triglyceride substrates in a form which interacts with lipases has been achieved by the dispersion of triglyceride in dilute solutions of detergents such as sodium oleate (7), gum acacia (8), bile salts (9), or Triton X-100 (1). Each procedure results in dispersions of different aggregation state, stability, and phase structure.

Because the substrate is in the form of multimolecular aggregates, the measurement of the initial rate kinetics of the reaction and the assessment of the role of effectors such as divalent metal ions and fatty acids have been difficult to establish (7). Studies of the saturation kinetics of pancreatic lipase (10) revealed complex saturation functions which could not be interpreted without the introduction of assumptions related to the aggregation state of the substrate. Any analysis of the saturation kinetics of substrates in micellar form is complicated by the possible interference of such factors as concentration-dependent alterations in micellar size,

* Mei-hui Teng is supported by a departmental predoctoral fellowship of the Department of Microbiology of Saint Louis University Medical School.

shape, and hydration. Investigations of the effect of amphipath concentration on the size of micelles have been performed with mixed dispersions of bile salts and neutral lipids (11, 12). Wallach (13) demonstrated that the charge of the detergent in a mixed micelle can affect the apparent pH optimum of lipase. Micelles of nonionic detergents may change in shape and in extent of hydration at high temperature and detergent concentration (14–16).

This report presents the results of our preliminary approach to the analysis of the saturation kinetics of beef liver lipase. Some of the properties of micellar dispersions of tripalmitin in Triton X-100, which determine the efficiency of lipase-catalyzed hydrolysis of triglycerides, have been assessed. Effects of Triton X-100 on the saturation kinetics of the enzyme have been analyzed, and the apparent K_m at zero Triton X-100 concentration has been determined by extrapolation procedures. In addition, the size of the micelles has been estimated by gel filtration techniques.

MATERIALS AND METHODS

Materials

Fresh beef liver was purchased from a local abattoir. Glycerol tripalmitate (tripalmitin) and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, Mo. Glycerol tripalmitate-1- 14 C was obtained from New England Nuclear Corp., Boston, Mass. The purity of the carrier and radioactive triglyceride was confirmed to be greater than 98%; further purification of these materials was performed as previously described (17). Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J., and Bio-Gel A-1.5 m from Bio-Rad Laboratories, Richmond, Calif.

Methods

Preparation of Soluble Beef Liver Lipase. The following procedures, chosen to yield maximal soluble lipase activity, were all performed at 4°C. Batches of sliced liver (150 g) were homogenized with 334 ml of 1.4×10^{-4} M EDTA and 0.352 M sucrose in 1.4×10^{-3} M Tris-HCl, pH 7.4, in a Waring Blendor for 2 min at maximum speed. Homogenates were adjusted to pH 7.4 by the dropwise addition of 1 N NaOH and they were then centrifuged for 30 min at 2000 g to remove nuclei and cell debris. Batches of the supernatant (600 ml) from this centrifugation were sonicated with a Biosonik II Sonifier (BP-II-5T probe) at a probe intensity of 70 in a 2-l beaker for 2 min in order to release soluble lipase activity from compartments in mitochondria and lysosomes. The sonicated supernatant was centrifuged for 30 min at 10,000 g to remove un-

disrupted mitochondria. The supernatant from this centrifugation was recentrifuged at 37,000 g for 2 hr to remove microsomes and submitochondrial particles. The supernatant from this centrifugation was removed with a 50-ml syringe and care was taken to transfer only the clear infranant solution. The lipase activity in this solution was used in all the experiments reported here. It was stable at -60°C for at least 3 months, and remained in the supernatant after centrifugation at 100,000 g for 2 hr.

Preparation of Standard Tripalmitin Dispersion. For each ml of final volume, 10 μ moles of glycerol tripalmitate-1- 14 C (specific activity indicated in figure legends) in chloroform was evaporated to dryness under a stream of filtered air at 60°C in a conical centrifuge tube. The tripalmitin was redissolved in 0.5 ml of petroleum ether (bp $35\text{--}50^\circ\text{C}$) and 187 mg of Triton X-100 was added. These components were alternately stirred on a Vortex mixer and heated to 60°C in a water bath until all the petroleum ether evaporated and a clear monophasic triglyceride in Triton X-100 was observed. This monophasic was diluted by the dropwise addition of distilled water at 60°C while vigorous stirring was maintained. The resulting water-clear dispersion was cooled to 23°C in a water bath.

Lipase Assay. Unless otherwise stated, the lipase was incubated for 10 min in a 1-ml final volume in the presence of 50 μ moles of sodium phosphate buffer, pH 6.65, 56 mg of Triton X-100, and 2 μ moles of glycerol tripalmitate-1- 14 C. The pH selected is at the midpoint of the shoulder of the pH profile of the activity. The reaction was stopped by the addition of 5 ml of isopropanol-heptane-1 N H_2SO_4 40:10:1, and the release of free fatty acid was measured as previously described (17) after the separation of radioactive free fatty acid from glycerides by extraction first into a heptane phase and then into an alkaline ethylene glycol phase.

Protein Assay. Protein was measured by the procedure of Lowry, Rosebrough, Farr, and Randall (18).

Gel Filtration Studies. In order to estimate micellar size and to assess changes in the size of micelles during aging, columns (0.65 \times 9.5 cm) containing Bio-Gel A-1.5 m were prepared in the usual manner and equilibrated at 37°C with an aged dispersion of tripalmitin in Triton X-100. The equilibration mixture was prepared by adding 20 ml of a dispersion of 10 μ moles/ml of glycerol tripalmitate in 280 mg/ml of Triton X-100 to 80 ml of a solution containing 0.0625 M sodium phosphate buffer, pH 6.65, and 0.025% sodium azide. This mixture was incubated at 37°C for 3 hr before it was applied to columns. In some instances, the micellar size was measured on Bio-Gel columns equilibrated with Triton X-100 dispersion prepared in exactly the same manner except for the omission of triglyceride. Experi-

ments described in the text regarding Sephadex G-200 gel filtration were performed with Sephadex columns equilibrated with the latter Triton X-100 dispersion. Sample application and elution were performed in the following manner. A standard glycerol tripalmitate-1-¹⁴C dispersion (specific activity 58,900 dpm/μeq) was diluted to 2 μmoles/ml triglyceride and 56 mg/ml Triton X-100. Aliquots of this preparation (50 μl) were placed on columns and eluted with the equilibration mixture at a flow rate of 0.35 ml/min. Fractions (0.08 ml) were collected and radioactivity was measured in scintillation vials containing 5 ml of scintillation liquid prepared as previously described (17). Columns were calibrated with 50 μl-aliquots of a solution containing 3 mg/ml of Dextran 2000, 3 mg/ml of Dextran 20, and 0.9 mg/ml of Vitamin B₁₂ before and after a series of runs.

RESULTS

Kinetics of Beef Liver Lipase with Freshly Diluted and with Diluted and Aged Dispersions of Tripalmitin as Substrate

As shown in Fig. 1A, when freshly diluted dispersions were used as substrate, the rate of triglyceride hydrolysis increased with time. The average rate in the first 10 min of incubation was 8.5 nmoles/min; from 10 to 22 min it was 9.8 nmoles/min; from 22 to 40 min it was 11.0 nmoles/min; and in the final 20 min plotted, it was 12.4 nmoles/min. The rate continued to increase for at least 90 min. Nonlinear kinetics with unaged substrate was observed with all batches of enzyme and substrate tested. The average range of duplicates was $\pm 3\%$, so these changes in the rate are significant. However, with aged substrate the rate was constant and was more than four times greater than the initial rate observed with unaged substrate. Barker and Jencks reported similar results for dispersions of a substrate for pig liver esterase (19). A linear relationship of enzyme concentration to rate of hydrolysis was obtained when aged substrate was used (Fig. 1B). The specific activity of this preparation of beef liver lipase measured in this way was 6.0 ± 0.2 nmoles/min/mg. The enzyme preparation is stable for at least 3 months at -60°C , and the specific activities of freshly thawed aliquots of this preparation were consistently within the range indicated.

Effect of Aging of Diluted Substrate Dispersions on the Saturation Kinetics of Beef Liver Lipase

In Fig. 2, saturation curves for the enzyme(s) are presented. The saturation kinetics of the enzyme preparation was measured directly after addition of the substrate to the reaction mixture and after the indicated times of aging of the substrate. When the kinetics is measured

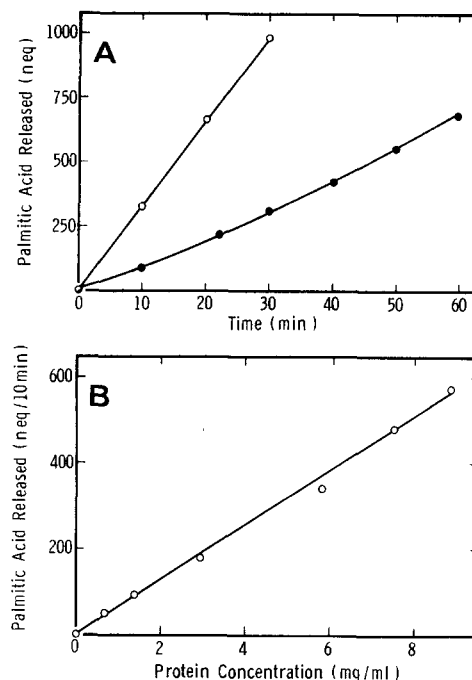


FIG. 1. Beef liver lipase-catalyzed hydrolysis of tripalmitin as a function of time and enzyme concentration. In Fig. 1A the amount of palmitic acid released from glycerol tripalmitate-1-¹⁴C (47,500 dpm/μeq) in unaged dispersions (●) and in aged dispersions (○) is plotted vs. time of incubation in the presence of enzyme. In Fig. 1B the amount of palmitic acid released from glycerol tripalmitate-1-¹⁴C of the same specific activity in aged dispersions is plotted vs. protein concentration. When the rate, with freshly diluted dispersions, was measured, 0.2 ml (5.9 mg of protein) of a preparation of soluble beef liver lipase was added to 0.5 ml of 0.1 M sodium phosphate buffer, pH 6.65, at 37°C . The reaction was started by the addition of 0.3 ml of a freshly prepared standard dispersion of tripalmitin in Triton X-100. When the rate of hydrolysis of diluted and aged dispersions of triglyceride was measured, the dispersion was added to the phosphate buffer and incubated at 37°C for 3 hr before the addition of enzyme.

after 3 hr of aging of substrate, a linear relationship between the reciprocals of velocity and substrate concentration is observed. However, complex kinetics are observed when measured with freshly diluted substrate. The velocity increases until it approaches a maximal value 2 to 3 hr after substrate aging begins. The equilibrium value is maintained for at least 2 hr. The relative extent and rate of increase in the velocity of hydrolysis is a linear function of the substrate concentration (Fig. 3).

Effect of Triton X-100 on the Saturation Kinetics of Beef Liver Lipase and on the Dispersion Aging Process

Linear reciprocal plots for all concentrations of Triton X-100 tested were obtained from initial rate measurements of the saturation of beef liver lipase with fully aged substrate dispersions (Fig. 4). Triton had no effect on the apparent V_{max} but markedly increased the apparent K_m . Fig. 5 is a plot of apparent K_m vs. Triton

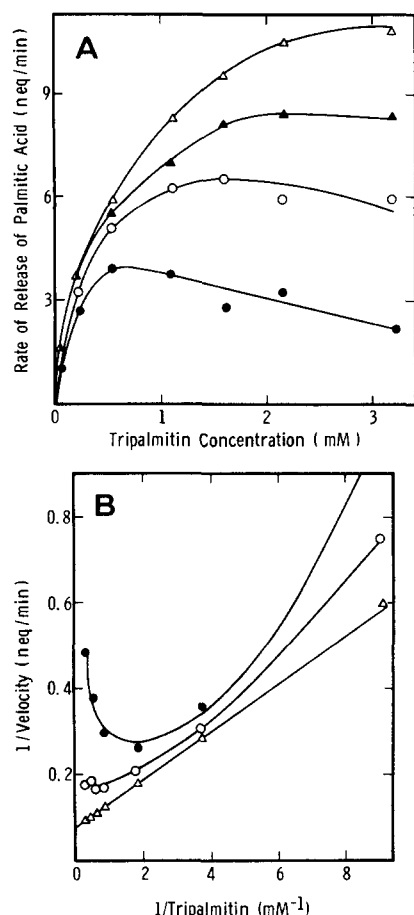


Fig. 2. Beef liver lipase-catalyzed hydrolysis of tripalmitin as a function of substrate concentration measured after aging of diluted dispersions. In Fig. 2A the rate of release of palmitic acid from glycerol tripalmitate-1- ^{14}C (70,500 dpm/ μeq) is plotted vs. the substrate concentration. In Fig. 2B the reciprocal of the rate of hydrolysis is plotted vs. the reciprocal of the substrate concentration. The saturation kinetics of beef liver lipase for tripalmitin was measured in a 1-ml reaction volume containing 1.47 mg of protein, the indicated amounts of tripalmitin, 56 mg of Triton X-100, and 50 μmoles of sodium phosphate buffer, pH 6.65, at 37°C. Incubations were carried out for 10 min. Substrate was incubated in the mixture at 37°C for the indicated periods of time before enzyme was added. The symbols in Fig. 2 represent the saturation curves obtained when diluted dispersions were not aged (\bullet), or when diluted dispersions were aged for 30 min (\circ), for 60 min (\blacktriangle), and for 180 min (\triangle).

X-100 concentration. The apparent K_m extrapolated to zero Triton X-100 concentration in this manner is $7.5 \times 10^{-5} \text{ M}$.

The results presented in Fig. 6 were obtained by the same procedure as those described in Fig. 2, except that tripalmitin concentrations were maintained at 1 mM and the Triton X-100 concentration was varied as indicated. The relative rate and extent of increase in the rate of hydrolysis are inversely proportional to the Triton X-100 concentration in the range measured (Fig. 6B).

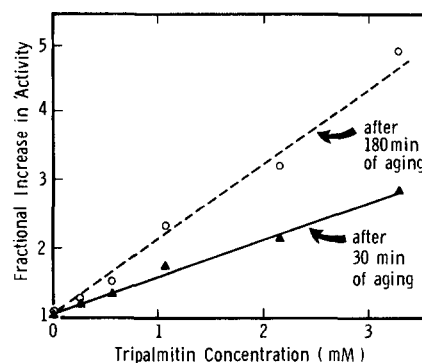


Fig. 3. Effect of time of aging of diluted tripalmitin dispersions on the velocity of hydrolysis of the substrate measured as a function of tripalmitin concentration. The fractional increase in the rate of release of palmitic acid from glycerol tripalmitate-1- ^{14}C (specific activity 70,500 dpm/ μeq) in aged preparations relative to that of unaged preparations is plotted vs. the substrate concentration in diluted dispersions. All experimental conditions are the same as in Fig. 2. Relative increase in the velocity of hydrolysis of the substrate after 30 min of aging of the diluted dispersions (\blacktriangle), and after 180 min of aging (\circ).

Estimation of Size of Mixed Micelles of Tripalmitin and Triton X-100

The size of the micelles was estimated by gel filtration on Sephadex and Bio-Gel columns as described in Methods. The micelles were completely excluded from Sephadex G-200. This indicates a micellar size greater than the molecular size of γ -globulin (which has a spherical diameter of 110 Å). The micelles were eluted from Bio-Gel A-1.5 m columns as a broad symmetrical peak which was retarded compared with the void volume marker, indicating a spherical diameter no greater than 400 Å (Fig. 7). This estimate of spherical radius is in good agreement with the determination of the micellar size of similar nonionic detergents by light-scattering techniques (14). There was a significant increase in the size of micelles aged in standard dispersions at 37°C for 4 hr (Fig. 7A). However (Fig. 7B), there was no significant change in the micellar size of dispersions which were diluted and aged in the manner described in Figs. 2 and 6.

Effect of pH on the Activity of Beef Liver Lipase

In Fig. 8, the pH profile of the lipase activity is presented. The pH profile was measured directly after the dilution of the substrate and after 3 hr of aging of the diluted substrate. The pH profile for the enzyme measured with aged substrate is characterized by a peak of activity at approximately pH 4.6 and a broad shoulder of activity in the range 5.6–7.6. There is no significant alkaline lipase activity peak. The pattern for the pH profile determined with freshly diluted substrate is similar, but the activity is markedly lower than that determined with aged substrate. The fractional increase in the ac-

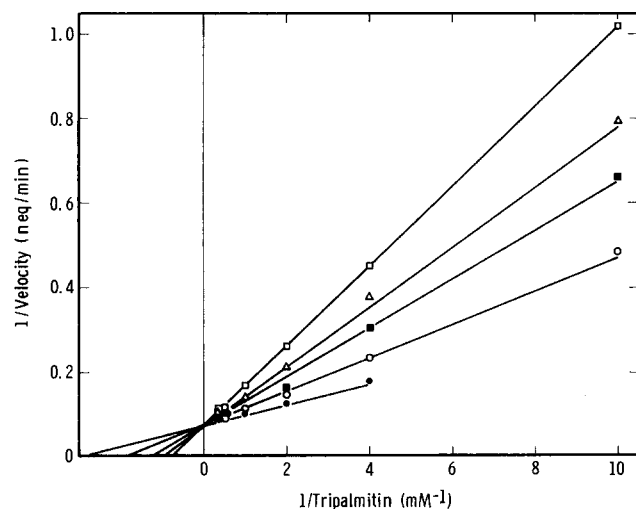


FIG. 4. Effect of Triton X-100 concentration on the saturation kinetics of the beef liver lipase-catalyzed hydrolysis of tripalmitin in aged dispersions. The results were calculated from measurements of the rate of hydrolysis of glycerol tripalmitate-1- ^{14}C (specific activity 58,900 dpm/ μeq) catalyzed by beef liver lipase in dispersions prepared at the indicated substrate and Triton X-100 concentrations and aged for 3 hr at 37°C before the addition of 0.05 ml of enzyme (1.68 mg of protein). The reaction was stopped after 20 min of incubation in the presence of enzyme and the release of free palmitate was measured as in Fig. 1. The symbols represent experimental points obtained at Triton X-100 concentrations of 18.7 mg/ml (\bullet), 37.4 mg/ml (\circ), 56.1 mg/ml (\blacksquare), 84.4 mg/ml (\triangle), and 112.8 mg/ml (\square). The lines drawn through these points were calculated with the aid of a programmed fitting procedure (20) as modified for a Hewlett-Packard desk computer.

tivity of enzyme determined with aged substrate over that determined with unaged substrate was independent of pH. The average value of this ratio was 5.3 ± 0.4 (SD) in the pH range of 3.6–7.6.

DISCUSSION

The finding that the gradual increase in the rate of hydrolysis of tripalmitin by beef liver lipase could be eliminated by aging the dispersion complex (Fig. 1) indicates that the nonlinear kinetics observed with freshly diluted dispersions was due to a change in the properties of the micelles during incubation. The simplest way to explain these changes is to postulate two forms for the micellar substrate: a form which is essentially nonproductive ("prosubstrate") and a form which is productive (effective substrate).

A plot of the reciprocal of velocity vs. the reciprocal of effective concentration should be linear if the enzyme exhibits Michaelis-Menten-type kinetics. The finding that the reciprocal of velocity is a linear function of the reciprocal of the added substrate concentration for fully aged dispersions (Fig. 2) indicates that the equilibrium concentration of effective substrate is proportional to the added substrate concentration. The probable cause of the

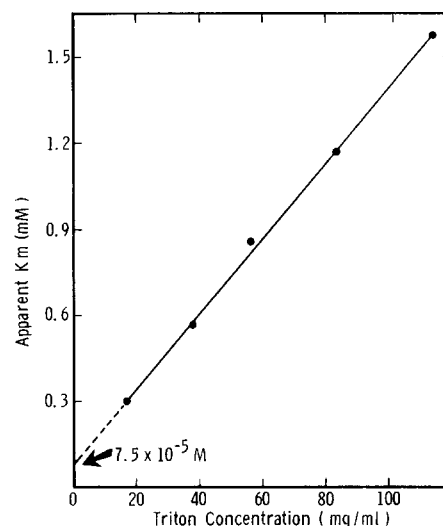


FIG. 5. Effect of Triton X-100 concentration on the apparent K_m of beef liver lipase for tripalmitin in aged dispersions.

complex Lineweaver-Burk plots of the reciprocals of velocity and added substrate concentration at zero time of aging of the substrate (Fig. 2) is that the effective substrate concentration is not proportional to the added substrate concentration under these conditions. Such considerations should be taken into account when complex saturation kinetics are observed for micellar substrates and may explain the apparent substrate inhibition observed by Mahadevan and Tappel (1).

One way to explain the effects of Triton X-100 on the saturation kinetics (Fig. 4) is by postulating a competition between Triton X-100 micelles and tripalmitin-Triton micelles for the substrate binding site on the enzyme. The diameter of the mixed substrate micelles has been estimated to be in the order of 100 Å. This finding, taken in conjunction with the reports that some lipases are lipoproteins (21, 22) gives credence to the possibility that in this system the triglyceride moves from the hydrophobic center of the mixed micelle into a hydrophobic region on the enzyme. Triton X-100 micelles could bind to this region and prevent access by mixed micelles containing substrate. An alternative explanation of the effect of Triton X-100 on the saturation kinetics is that the fraction of added substrate which is in the form of effective substrate at equilibrium is inversely proportional to the Triton X-100 concentration (Fig. 6). This possibility should be taken into consideration when apparent competitive effects are observed with micellar substrates, and it may play a role in the effects of esters on the saturation kinetics of rat liver lipase observed by Okuda and Fujii (21).

It is possible that more than one enzyme is present in the preparations described in this report. Further, the interaction of the substrate with a grouping of different

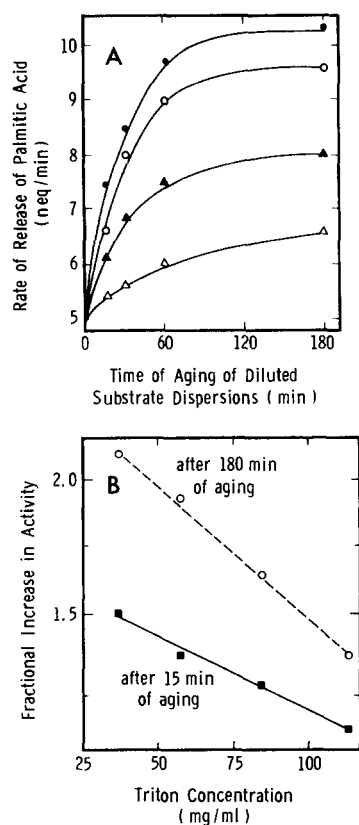


FIG. 6. Effect of time of aging of diluted tripalmitin dispersions on velocity of hydrolysis of substrate measured as a function of the Triton X-100 concentration. In Fig. 6A the rate of release of palmitic acid from glycerol tripalmitate-1- 14 C (specific activity 58,900 dpm/ μ eq, final concentration 1 mM) catalyzed by beef liver lipase is plotted vs. the time of aging of diluted substrate dispersions. Conditions of aging, incubation, and assay are described in Fig. 2. The symbols in Fig. 6A represent the aging curves obtained at final Triton X-100 concentrations of 37.4 mg/ml (\bullet), 56.1 mg/ml (\circ), 84.4 mg/ml (\blacktriangle), 112.8 mg/ml (\triangle). In Fig. 6B the fractional increase in the rate relative to that of unaged preparations is plotted as a function of the Triton X-100 concentration. The symbols in Fig. 6B represent the relative increase in the productivity of the substrate after 15 min of aging of the diluted dispersion (\blacksquare) and after 180 min of aging (\circ).

lipases might be essential for the sensitivity of the activity to alterations in the substrate. The shape of the pH profile is suggestive of the presence of at least two lipases. However, the extent of sensitivity of the activity to these alterations is independent of the pH of aging of the substrate or the pH of assay of the enzyme. Therefore, if a grouping of lipases is required for the phenomenon, the structure of the grouping must be insensitive to pH. The final resolution of this problem will come from the purification of the lipase(s). At the present time, we have succeeded in purifying the activity 12-fold. No physical or chemical evidence has been obtained for more than one lipase in this preparation.

At the present time, the properties of the substrate undergoing a conversion from "prosubstrate" to effective substrate are not known. The size and distribution

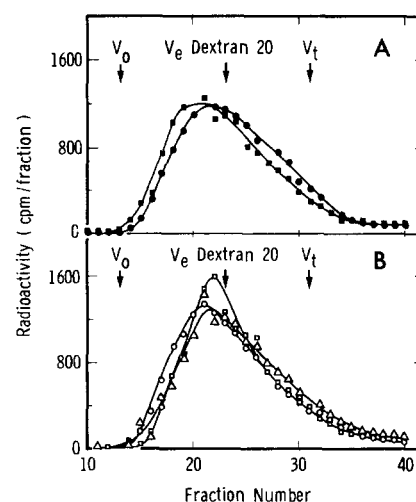


FIG. 7. Estimation of micellar size of tripalmitin dispersion complexes on Bio-Gel A-1.5 m. This figure illustrates the patterns of elution of glycerol tripalmitate-1- 14 C dispersions (50 μ l/sample, 58,900 dpm/ μ eq) from a Bio-Gel A-1.5 m column equilibrated with an aged, unlabeled tripalmitin dispersion of the same tripalmitin concentration (2 μ moles/ml) and Triton X-100 concentration (56 mg/ml) as the sample. The arrows, from left to right, indicate void volume (V_0), elution volume (V_e) of yellow dextran 20 (mol wt 20×10^3), and total volume (V_t) of the column. The symbols in Fig. 7A represent the patterns obtained with samples which were applied to the column immediately after they were prepared from unaged standard dispersions (\bullet) and with samples which were applied to columns immediately after they were prepared from standard dispersions which had been incubated at 37°C for 4 hr (\blacksquare). The symbols in Fig. 7B represent the patterns obtained with samples prepared from unaged standard dispersions and incubated at 37°C for 30 min (\circ), 60 min (\square), and 120 min (\triangle) before they were applied to the column.

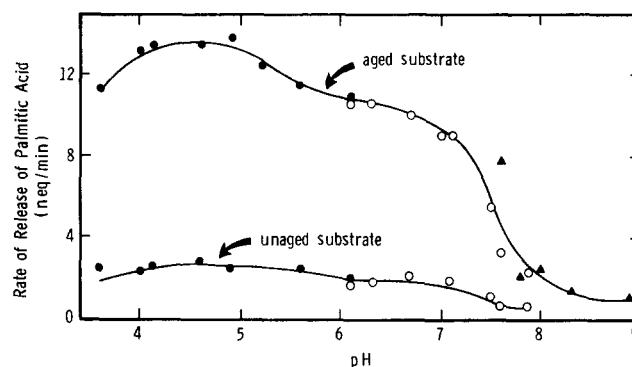


FIG. 8. Effect of pH on the activity of beef liver lipase. The rate of release of palmitic acid from glycerol tripalmitate-1- 14 C in aged and unaged dispersions is plotted vs. pH. Conditions of incubation, aging, and assay were the same as in Fig. 1, except that 1.47 mg of protein was used and the buffers were either 0.05 M sodium acetate-acetic acid (\bullet), 0.05 M sodium phosphate (\circ), or 0.05 M Tris-HCl (\blacktriangle), at the indicated pH. Reactions were stopped after 10 min of incubation in the presence of enzyme.

of the micelles do not change significantly with time of aging after dilution (Fig. 7). This finding is in agreement with the report of Borgström (11) that mixed micelles of triglycerol ethers and bile salts are not affected by concentration changes above the critical

micellar concentration for the detergent. Furthermore, the decomposition of "prosubstrate" to smaller micelles should be first order in "prosubstrate" concentration. However, a plot of the log of the "prosubstrate" concentration vs. time of aging calculated for data in Fig. 3 is not a straight line. In some cases demicellization following dilution has been found to occur with a rate comparable to that of the aging process reported here (23, 24). The extent of the demicellization process should decrease as the dilution of Triton X-100 decreases. If it is assumed that the effective substrate is demicellized triglyceride stripped of Triton X-100, then the data in Fig. 6 would be in consonance with this hypothesis. However, there are many other possibilities, including alterations in the hydration and shape of the micelles, which could explain the reported observations.

The use of micellar dispersions of triglyceride in detergent as substrates for lipase has many advantages. First, they approximate the form of endogenous substrate in vivo. The study of the factors which prevent their conversion to emulsion droplets may have significance for the elucidation of the processes which produce fatty livers, the fatty degeneration associated with hepatomas, and the fatty plaques of atherosclerotic lesions. Second, the dispersions can be prepared in a manner which gives reproducible activity at low saturating triglyceride concentration relative to emulsions (Fig. 1). This decreases some of the assay interference associated with the use of high substrate concentrations. Third, appropriate extrapolation procedures are available to nullify effects of detergent on the enzyme (Fig. 5). Finally, the use of enzymes as probes of micellar properties may be a valuable tool for the investigation of the physical chemistry of lipids.

The authors would like to express their appreciation for the expert technical assistance of Miss Dianne Farris.

This work was supported by U.S. Public Health Service Grant AM13516.

Manuscript received 24 July 1970; accepted 14 January 1971.

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