HYDROLYSIS OF MONOMOLECULAR FILMS OF TRIOCTANOIN BY PORCINE PANCREATIC LIPASE

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Monomolecular films of trioctanoin were hydrolyzed by lipase (EC 3.1.1.3). The rate of hydrolysis was linear with enzyme concentration at all film pressures above approximately 5 dynes cm^{-1}, including the collapse pressure. Compression of the remaining substrate to the collapse pressure after hydrolysis or redeposition of triglyceride restored the maximum rate of hydrolysis.

Comparison of the rates of hydrolysis of trioctanoin analogs modified in the glycerol moiety of the triester indicated that pancreatic lipase required a primary alcohol at position 1. If no substituent was present at position 2, or if there was disubstitution, the action of lipase was greatly reduced. Either an oxygen or amino function on position 2 was necessary. Three carbon atoms were required in the alcohol portion of the substrate, but an ester function was not required at position 3.

Porcine pancreatic lipase (EC 3.1.1.3) preferentially hydrolyzes insoluble triglycerides at the oil/water interface in emulsions (1,2). The hydrolytic action of a soluble enzyme on an insoluble substrate could provide a simplified system with which to study lipid-protein interactions. An advantage of such a system is that one could directly relate the hydrolysis of the ester to a surface property of the lipid substrate.

The rate of hydrolysis of a triglyceride by lipase is typically determined by the rate of release of hydrogen ion by titration (3). To achieve a conveniently high rate of reaction, an emulsion of the substrate is often prepared with the aid of emulsifiers (3,4). However, correlation of the rates of hydrolysis of substrates and substrate analogs with substrate specificity of lipase is ambiguous because of the variability of emulsions.

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Therefore, a second objective was to circumvent the difficulty which results from the use of emulsions. The interaction of lipase with monomolecular films of a typical substrate was investigated and found to be a satisfactory system to study hydrolysis of the ester substrate without the difficulties presented by emulsifiers. Dawson developed a system with which the hydrolysis of lecithin monolayers by phospholipase was examined by changes in surface pressure, surface potential, and surface radioactivity (5).

Materials and Methods

Glycerol-^{14}C-trioctanoate (8.2 mCi/mmole) was obtained from New England Nuclear. Trioctanoin was obtained from Distillation Products, Inc., and redistilled before use. Analogs of trioctanoin were prepared by interaction of octanoyl chloride with the appropriate alcohol in anhydrous pyridine and the esters purified by distillation. All substrates were >99% pure by gas-liquid chromatography. Mass spectra have been obtained with an LKB 9000 gas chromatograph-mass spectrometer to confirm the structures of the trioctanoin analogs. The syntheses will be reported in detail elsewhere.

Porcine pancreatic lipase was purified to ca. 75% homogeneity (gel electrophoresis) from an acetone powder by a modification of the procedure of Verger et al. (6).

Changes in film pressure were measured with a Cahn recording electrobalance calibrated in dynes cm^{-1}. The sensor was lightly sanded platinum foil (perimeter of 1.0 cm) suspended from the "C loop" of the balance. The reaction vessel was machined from polytetrafluoroethylene with inside dimensions of 100 x 34.5 x 3.0 mm.

The aqueous phase (16 ml), 5 mM Tris, pH 8.1 contained 0.2 M NaCl and enzyme in the amounts indicated. The surface of the aqueous phase was swept with a plexiglass barrier to remove surface contaminants.

For determinations of force-area curves, aliquots of trioctanoin (2-4 nmole) dissolved in benzene were applied from a microliter syringe directly onto the swept surface. After evaporation of the benzene (5-10

sec), the film was compressed by moving the barrier with a motorized drive at a constant rate (0.43 cm/min) until the collapse pressure was achieved.

To examine enzymic hydrolysis of a monolayer, an amount of substrate in excess of that required to form a monolayer (10-20 nmoles) was applied in benzene to the lipase solution. After attaining the collapse pressure (15-60 sec), the film pressure decreased as the monofilm was hydrolyzed. The maximum rate of hydrolysis was measured at pressures near the collapse pressure. All experiments were performed at room temperature (25°).

To determine the products of enzymic hydrolysis of a trioctanoin monolayer, glycerol-^{14}C-trioctanoate (12 nmoles, 100 nCi) was spread on the surface of the

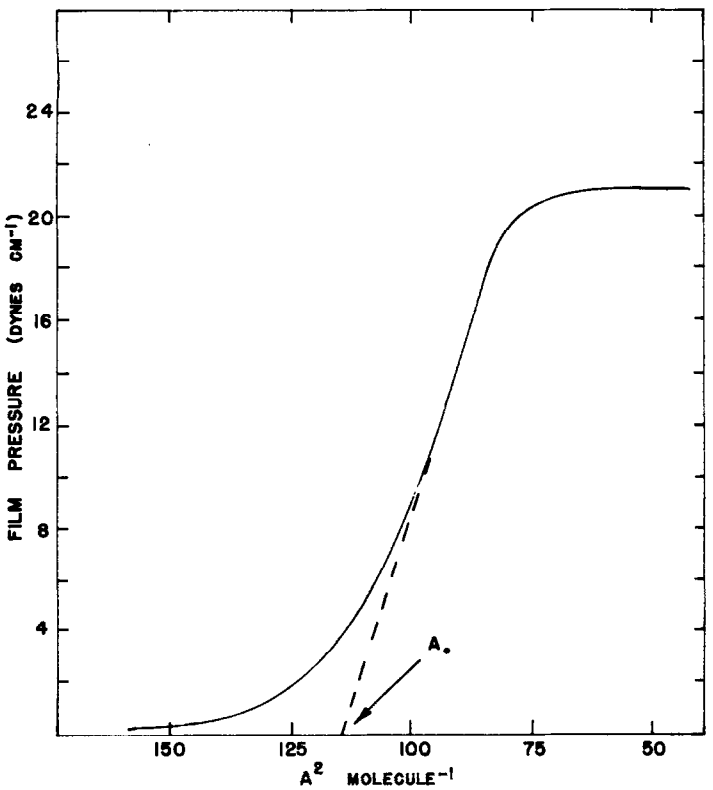

FIGURE 1

The force-area curve for trioctanoin was determined under conditions described in Materials and Methods after spreading trioctanoin (3.0 nmoles in 10 μl of benzene) onto the swept aqueous surface.

reaction mixture containing lipase (10 μg of protein). Lipase was omitted from an identical vessel as the control. After the hydrolysis had proceeded for two minutes, the reaction mixture was extracted with 10 ml of benzene and the organic phase was reduced in volume. The products were separated by thin-layer chromatography on silica gel in ether-hexane (4:1). The radioactivity of 0.5 cm sections was determined in a liquid scintillation counter. For reference, nonlabeled mono-, di-, and triglycerides of octanoic acid were chromatographed and visualized by iodine vapor.

Results and Discussion

The force-area curve of trioctanoin is shown in Figure 1. Changes in the

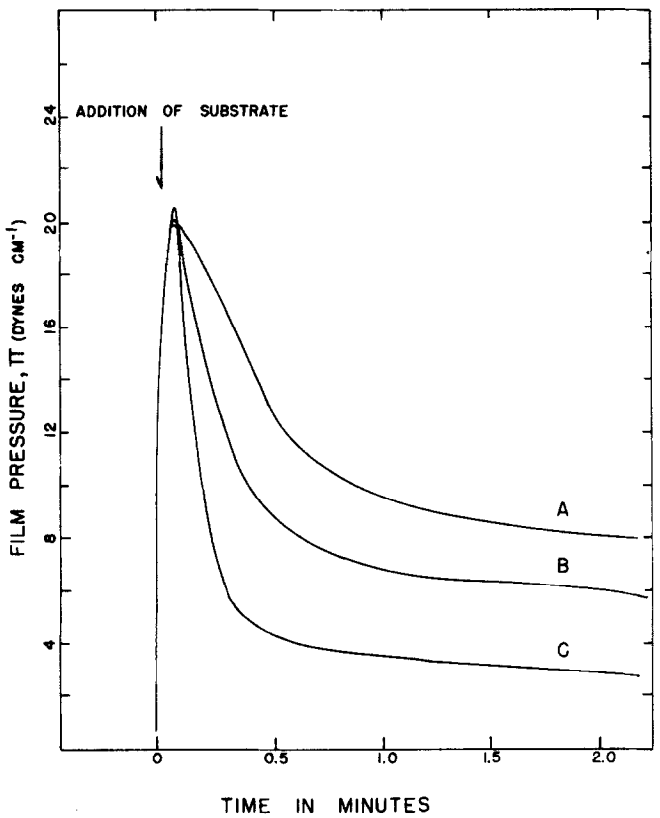

FIGURE 2

For the determination of the rate of enzymic hydrolysis of a trioctanoin monolayer, the reaction mixture described in Materials and Methods was supplemented with partially purified lipase in (A) 10 μg, (B) 20 μg, and (C) 50 μg of protein.

film pressure were linear with changes in area in the range of 10-18 dynes cm^{-1}. The collapse pressure was approximately 21 dynes cm^{-1}. The "limiting area" per molecule, A_o, determined by extrapolation to zero force, was 114 A^2 per molecule.

Trioctanoin films were hydrolyzed when lipase was included in the subphase as described in Figure 2. The rate of hydrolysis was determined from the slope near the collapse pressure where decrease in film pressure was linear with respect to time. Measurable hydrolysis occurred until the film pressure decreased to 3-8 dynes cm^{-1}. The rate of hydrolysis was proportional to the amount of lipase added to the subphase as shown in Figure 3 and was linear throughout a 100-fold range of lipase concentrations.

In the experiment described in Figure 4, enzymic hydrolysis of trioctanoin

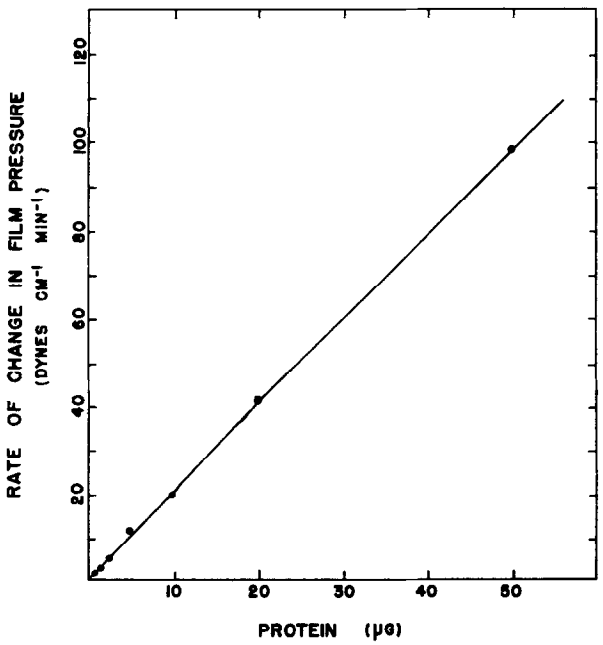

FIGURE 3

The rate of enzymic hydrolysis of a trioctanoin monolayer was measured as a function of the amount of partially purified lipase added to the aqueous phase under conditions described in Materials and Methods. The points at 10, 20, and 50 µg protein were obtained from curves A, B, and C, respectively, shown in Figure 2.

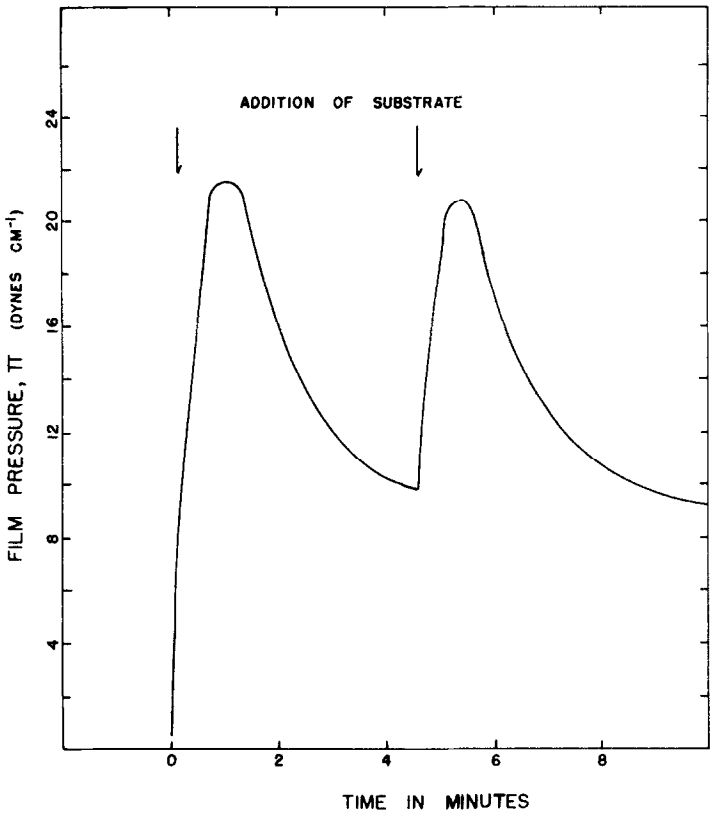

FIGURE 4

After the first addition of trioctanoin (12 nmoles in 4 μl of benzene), additional trioctanoin (6 nmoles in 2 μl of benzene) was added at the indicated time. The subphase contained partially purified lipase, 10 μg of protein. Other conditions are described in Materials and Methods.

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monolayers was allowed to proceed until the rate of hydrolysis was approximately 10% of the maximum rate. Additional trioctanoin was then added to the surface to restore the film pressure to the collapse pressure. Hydrolysis of the monolayer again proceeded at a rate near the original rate. In a similar experiment, in Figure 5, the hydrolysis of the monolayer was allowed to proceed until the rate was 10% of the original rate. The barrier was moved to compress the film until the collapse pressure was again achieved, whereupon the hydrolysis of the monolayer proceeded at a rate essentially the same as the original rate.

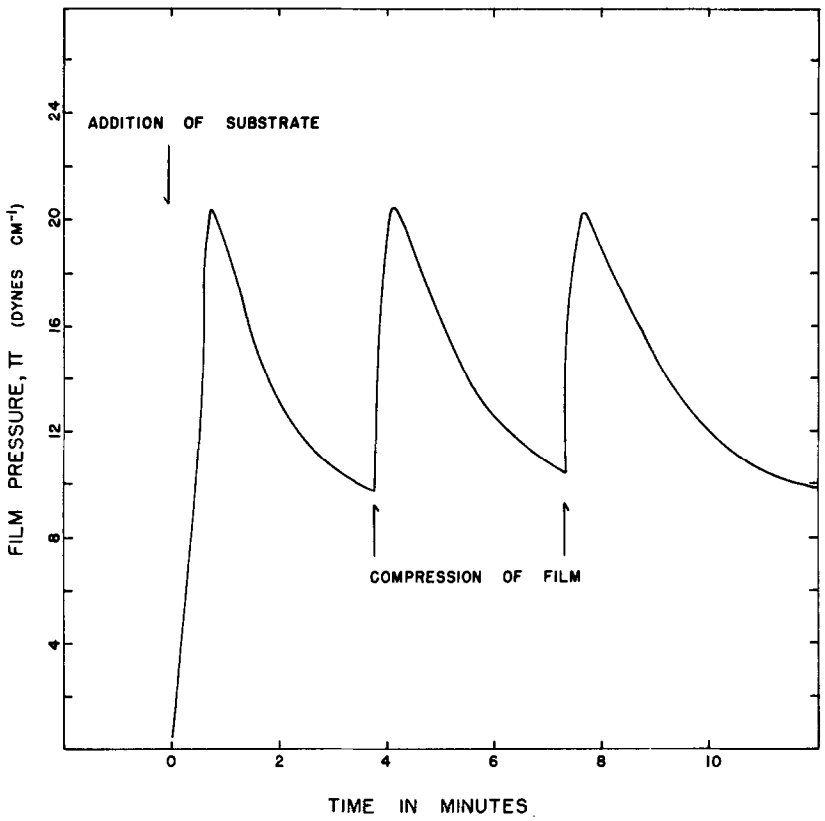

FIGURE 5

After the enzymic hydrolysis of the trioctanoin monolayer had proceeded to the time indicated, the remaining film was compressed to the collapse pressure by advancing the movable barrier. The conditions were identical to those described in Figure 4 and in Materials and Methods.

The structural requirements for the hydrolytic action of pancreatic lipase on triglycerides are presented in Table I. Branching at position 2 of the acyl function, as in tri-(2-ethylhexanoyl)-glycerol, prevented hydrolysis. Replacement of a hydrogen atom by a methyl group on either position 1 (dioctanoyl-2,3-butanediol) or position 2 (trioctanoyl 2-methylglycerol) of the alcohol function greatly reduced the ability to be hydrolyzed. The rate of cleavage of the primary ester in the 1,3-diglyceride, which has a free hydroxyl group on position 2, was reduced only one-half. If no substituent at carbon-2 (dioctanoyl-1,3-propanediol) was present or if there was

TABLE I

Hydrolysis of Monolayers of Trioctanoin Analogs by Lipase

	Substrate	Rate of Change in Film Pressure (dynes cm^{-1} min^{-1})	Percent of Control
1.	Trioctanoyl glycerol (Trioctanoin)	27.1	(100)
2.	Tri-(2-ethylhexanoyl) glycerol	<0.1	<0.3
3.	Dioctanoyl-2,3-butanediol	0.4	2
4.	Trioctanoyl 2-methyl-glycerol	0.1	0.3
5.	1,3-Dioctanoyl glycerol	11.0	41
6.	Dioctanoyl-1,3-propanediol	1.7	6
7.	Dioctanoyl-2,2-dimethyl-1,3-propanediol	<0.1	<0.3
8.	Dioctanoyl-2-amino-1-propanol	14.8	55
9.	Dioctanoyl-2-methyl-1,3-propanediol	0.3	1
10.	Dioctanoyl-1,2-propanediol	21.7	80
11.	1,2-Dioctanoyl-3-O-methyl glycerol	24.6	91
12.	Dioctanoyl-1,2-ethanediol	4.5	17

The reaction mixture contained 5 mM Tris, pH 8.1, and 0.2 M NaCl with partially purified lipase, 10 μg of protein. After sweeping the surface with a movable barrier, aliquots of benzene (2-4 μl) containing the appropriate substrate (10-20 nmoles) were added in excess of that amount required to achieve the collapse pressure of the oil. Each rate of hydrolysis was determined as the slope of the progress curve at film pressures just below the collapse pressure. Each value is the average of at least two determinations.

disubstitution (dioctanoyl-2,2-dimethyl-1,3-propanediol), little hydrolysis occurred. If the ester function at position 2 was replaced with an amide as in dioctanoyl-2-amino-1-propanol, hydrolysis of the ester at position 1 still occurred at an appreciable rate. The lack of hydrolysis of dioctanoyl-2-methyl-1,3-propanediol in contrast to the appreciable hydrolysis of 1,3-dioctanoyl glycerol and dioctanoyl-2-amino-1-propanol suggested that a heteroatom - either oxygen or nitrogen - was required at the 2 position of the glycerol moiety and that there was not simply a steric requirement at

this position. The differences in the rate of hydrolysis of dioctanoyl-1,2-propanediol and dioctanoyl-1,2-ethanediol indicated that three carbon atoms were required in the alcohol portion of the substrate for rapid hydrolysis by lipase, although an ester function was not necessary, as evidenced by the high rate of hydrolysis of the 3-O-methyl ether of 1,2-dioctanoyl glycerol.

That the observed decrease in film pressure results from the hydrolytic

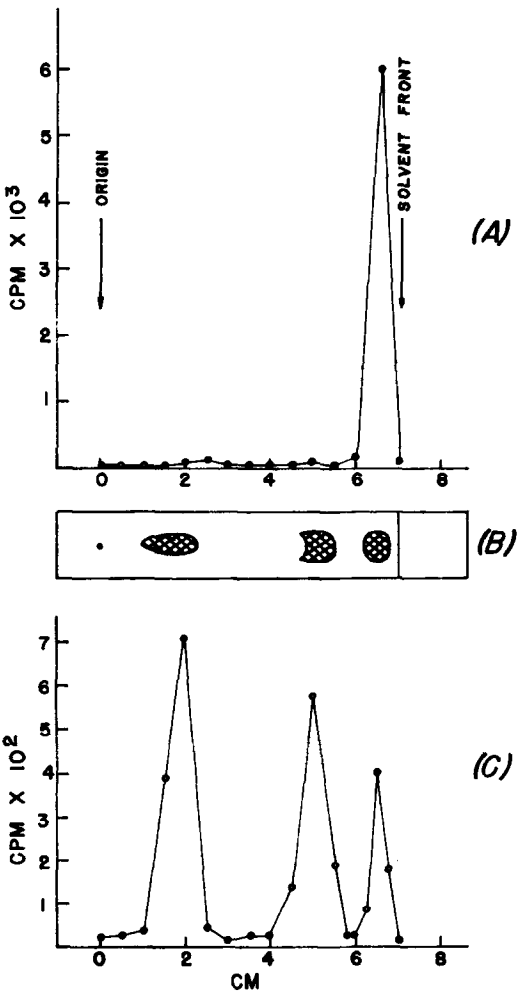

FIGURE 6

The products of the enzymic hydrolysis of a monolayer of ^{14}C-trioctanoin were determined as described in Materials and Methods. In (A), the reaction mixture contained no lipase. In (B), mono-, di-, and trioctanoyl glycerates were visualized by iodine vapor. In (C), the reaction mixture contained lipase (10 μg of protein).

action of lipase is supported by the following experiments. (a) The decrease in film pressure was linear with time (Figure 2) and with enzyme concentration (Figure 3). (b) Hydrolysis of the monolayer occurred with both crude and partially purified lipase. The enzymatic activity determined by hydrolysis of the monolayer and by titrimetry was a constant ratio. (c) Substitution of 100 μg of either fatty acid poor bovine serum albumin or trypsin did not cause a decrease in film pressure. (d) Both possible products of trioctanoin - mono- and dioctanoin - were identified as the result of lipase action (Figure 6)

The decrease in the film pressure also suggested that the products did not affect the subsequent rate of reaction at lower film pressures. This is consistent with the following observations. The initial rate of hydrolysis was restored by additional substrate (Figure 4) or by compression of the film to the original collapse pressure (Figure 5). Conversion of the initial diglyceride product to the monoglyceride occurred (Figure 6).

The results of this investigation demonstrate a simple and precise method for the determination of the interaction of lipase with monomolecular films of substrates and substrate analogs at the air/water interface. The measurement of changes in the film pressure provide a convenient method for the comparison of the rates of hydrolysis of a series of substrates. This method requires only a subphase of compatible environment for lipase, a substrate which is sufficiently insoluble in the subphase to form a monolayer, and that at least one of the products be soluble in the subphase. A variety of modifications of the system - such as changes in the ionic environment - are possible. The mode of action of lipase can be studied without the ambiguities introduced by emulsions and emulsifiers.

Acknowledgments

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References

1. Sarda, L. and Desnuelle, P., Biochim. Biophys. Acta 30, 513 (1958).
2. Entressangles, B. and Desnuelle, P., Biochim. Biophys. Acta 159, 285 (1968).
3. Desnuelle, P. and Savary, P., J. Lipid Res. 4, 369 (1963).
4. Bier, M., Methods in Enzymology, Colwick and Kaplan, Eds., Volume I, 627 (1955).
5. Dawson, R. M. C., Methods in Enzymology, Lowenstein, Ed., Volume 14, 633 (1969).
6. Verger, R., De Hass, G. H., Sarda, L. and Desnuelle, P., Biochim. Biophys. Acta 188, 272 (1969).
7. Davies, J. T. and Rideal, E. K., Interfacial Phenomena, Academic Press New York (1963), Chapter 5, pp. 217-281.