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ACTION OF PANCREATIC LIPASE ON AGGREGATED GLYCERIDE MOLECULES IN AN ISOTROPIC SYSTEM

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SUMMARY

NaCl enhances the hydrolysis by pancreatic lipase (glycerol-ester hydrolase, EC 3.1.1.3) of concentrated isotropic solutions of triacetin, tripropionin and 1,3-dibutyrin. These short chain glycerides spontaneously associate in concentrated solutions containing NaCl to form molecular aggregates on which lipase is active. Lipase is therefore able to act, not only as believed earlier on emulsified ester particles, but also on aggregates of a much smaller size in an isotropic system. With triacetin and tripropionin, the maximal rates of hydrolysis are similar for aggregates and particles of the same substrate.

Preliminary experiments by light scattering suggest that, at 25° in the presence of 0.1 M NaCl, tripropionin forms aggregates of a rather uniform size containing about 15 monomers. Triacetin aggregates are smaller. The mechanism by which substrate aggregation induces lipolysis is still unknown.

INTRODUCTION

The interactions of pancreatic lipase (glycerol-ester hydrolase, EC 3.1.1.3) with insoluble long chain glycerides have been extensively studied in recent years[1,2]. It was found[2] that the initial rate of lipolysis in an emulsion stabilized by deoxycholate is controlled by the area of the glyceride–water interface in one volume unit of emulsion. All other factors being constant, this "interface concentration" plays the same role as the substrate concentration in ordinary aqueous solutions. When it is increased for a fixed amount of lipase, more enzyme molecules are adsorbed at the interface and catalyze the reaction. Thus, the reaction rate goes up and reaches a maximal value (v_{max}) corresponding to complete adsorption. Similarly, a K_m (emulsion) of lipase can be defined as the concentration of the interface for which the rate is $v_{max}/2$.

An investigation of water-soluble short chain glycerides has also been made[1]. Aqueous, optically clear solutions of these substrates were found to be poorly hydrolyzed by purified lipase even when the substrate concentration was high. In contrast, a considerable rise in activity was observed when, by adding more substrate, emulsified particles began to appear in the oversaturated solutions. It was concluded that lipase

was exclusively able to catalyze the hydrolysis of substrate molecules located at the interface of emulsified particles, after previous adsorption to this interface.

It will be shown that, when increasing amounts of short chain glycerides are added to a fixed volume of water containing 0.1 M NaCl, these glycerides appear in an intermediary aggregated form between the molecularly dispersed solution and the heterogeneous emulsion. In other words, two transitions can be observed with these compounds, as with amphipaths. The first transition is characterized by the formation of aggregates which can be detected by their action on the optical properties of some dyes and by light scattering. An important factor is that, after this first transition, the system remains homogeneous or isotropic. In contrast, the other transition, corresponding to what is currently called the saturation, is characterized by the appearance of a second phase forming relatively large emulsified particles. The concentration at which this transition occurs (the saturation point) is experimentally determined by means of the steep increase in the absorbance of the system at 400 mμ, due to the existence of an interface separating the two phases.

Results obtained are consistent with the view that pancreatic lipase is able to act, not only as believed earlier on emulsified particles, but also on the much smaller aggregates mentioned above. HOFMANN AND BORGSTRÖM[3] had already observed that rat pancreatic juice can hydrolyze micellar solutions of 1-mono-olein in taurodeoxycholate, and they attributed this kind of activity to lipase. However, MORGAN *et al.*[4] later found that another, still unidentified enzyme in the juice was responsible for the hydrolysis of the micellar glyceride. In the studies made here, no amphipathic molecules were used for the stabilization of emulsified particles or aggregates, so that results obtained with emulsions and isotropic solutions are directly comparable. The physical state of the substrate is merely modified by varying the concentration and ionic strength of the system. This can only be done with short-chain derivatives having a distinct solubility in water.

TECHNIQUES

Purification of lipase and activity determinations

Lipase was purified[5] from aqueous extracts of fresh porcine pancreas by several precipitations by ammonium sulfate and acetone followed by chromatography on DEAE-cellulose. The specific activity (number of lipase units/mg protein (Lowry)) was about 4000 for all preparations.

Initial lipolysis rates were measured titrimetrically in a Radiometer pH-stat at 25° and pH 7.0 on 15 ml of substrate solutions or emulsions in 0.1 M NaCl. The emulsions were prepared as follows. The 0.1 M NaCl solution was placed in the beaker of the pH-stat (titrating device TTA3), a known excess of glyceride was added and the mixture was agitated for 30 min at 1400 rev./min. Agitation was maintained after addition of lipase. In contrast to long chain triglycerides, short chain compounds formed rather stable emulsions under these conditions in the absence of emulsifiers. The average size of the emulsified particles appeared to be similar in all assays, as shown by the reproducibility of the results and by the fact that linear Lineweaver–Burk plots could be obtained (see later).

In all experiments, the flow rate of 0.02 M NaOH was recorded for a few minutes in order to evaluate the spontaneous hydrolysis of the substrate. Then lipase was added

and the flow rate was again recorded as a function of time. Enzyme activity was calculated by difference and expressed as a percentage of the rate observed at 25° and pH 9.0 with the same amount of lipase and an excess of emulsified triolein under the conditions worked out for routine assays of the enzyme[6]. It was verified that the liberated short chain fatty acids (acetic, propionic, butyric) were completely ionized at pH 7.0. Thus, the lipolysis rates could be directly calculated from the responses of the pH-stat.

Purification of substrates and evaluation of their solubility

Commercial triacetin and tripropionin were purified by distillation or chromatography on Florisil[7], respectively. Tripropionin was eluted by a 75:25 hexane–diethyl oxide mixture. For 1,3-dibutyrin, 2 moles of butyryl chloride were condensed with 1 mole of glycerol in the presence of pyridine. Purification was achieved by chromatography on silicic acid[8]. After the column had been washed by a 80:20 hexane–diethyl oxide mixture, 1,3-dibutyrin was eluted by a 35:65 mixture of the same solvents. Triacetin and tripropionin were found to be pure by thin-layer chromatography. 1,3-Dibutyrin contained about 5% of the 1,2 isomer.

The solubility of the compounds in 0.1 M NaCl at 25° was measured by adding increasing amounts of glycerides to a fixed volume of solvent. The mixtures were vigorously shaken for 60 min and their absorbance at 400 mμ was measured against a 0.1 M NaCl solution in a Beckman Spectrophotometer equipped with a thermostated cell compartment. Extrapolation to the base line of the absorbance *versus* concentration plot gave the concentration value at which the saturation was attained. These values were 7.20 g, 0.25 g and 1.04 g/100 ml for triacetin, tripropionin and 1,3-dibutyrin, respectively. The solubility of triacetin in water at 20° is given in the *Handbook of Chemistry and Physics* (41st ed., p. 1015) as 7.17 g/100 ml.

Detection of molecular aggregates

The existence of molecular aggregates in the solutions was detected by a series of techniques currently used for the evaluation of the critical micellar concentration of amphipathic molecules.

Solubilization of Sudan III (ref. 9)

This water-insoluble dye is partly solubilized in the hydrophobic moiety of micelles. Glyceride solutions in 0.1 M NaCl (5 ml) were shaken for 12 h at 25° with 10 mg Sudan III in small stoppered vials containing some glass beads. A blank without glyceride was run simultaneously. After centrifugation, the absorbance of the clear solutions was read at 505 mμ.

Spectral shift with benzopurpurin 4 B (ref. 10) and iodine[11].

Interactions of micelles with some water-soluble dyes induce a spectral shift which can be used for differential spectrophotometry determinations. Two solutions in 0.1 M NaCl were prepared. The first was 0.9 saturated in glyceride and contained the dye (benzopurpurin 50 mg/l; iodine 60 mg/l). The second contained the dye only. Different volumes of the solutions were mixed in order to vary the glyceride concentration for the same concentration of the dye. The absorbance of the samples against the dye solution was measured within 1 h with a Zeiss spectrophotometer Model PMQ II. The wavelength used was that corresponding to the maximal spectral shift (510 mμ for benzopurpurin and 355 mμ for iodine).

RESULTS

The assays described below were performed with three short chain glycerides, triacetin, tripropionin and 1,3-dibutyrin which give optically clear (isotropic) aqueous solutions and also emulsions when the solutions are oversaturated. An important point is that, in contrast to long chain triglycerides, short chain glycerides form stable emulsions when shaken with water in the absence of emulsifiers.

Effect of NaCl on triacetin hydrolysis in an isotropic system

Curve I in Fig. 1 shows that addition of NaCl has little effect on the hydrolysis rate of a dilute (0.2 saturated) triacetin solution by lipase. In sharp contrast however, NaCl accelerates the hydrolysis of a concentrated (0.9 saturated) solution of the same substrate (Curve II, Fig. 1). This effect is maximal (about 4-fold) for a 0.05 molarity in NaCl. All subsequent assays of this series were carried out in 0.1 M NaCl.

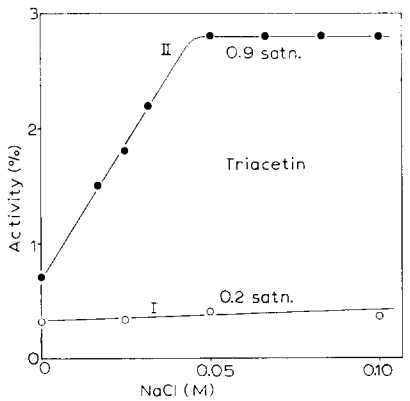


Fig. 1. Effect of NaCl at two triacetin concentrations. The concentration of triacetin was 1.44% (0.2 saturation) for Curve I and 6.50% (0.9 saturation) for Curve II. For each assay 22 lipase units were used. Rates are given in per cent of the maximal rate observed with the same amount of lipase and an excess of a triolein emulsion.

Effect of substrate concentration on the activity of lipase in an isotropic system

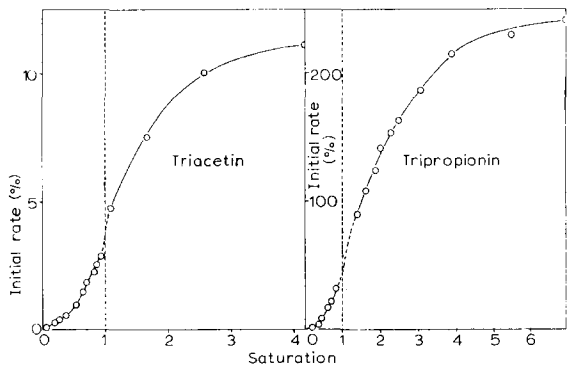
The results presented in Fig. 1 suggest that lipase activity in an isotropic solution containing 0.1 M NaCl strongly depends on the concentration of the substrate. Fig. 2 shows the variation with triacetin and tripropionin as substrates. Each diagram is divided into two parts by a vertical dashed line indicating the saturation point. On the left of this line lipase is acting on isotropic solutions of increasing concentrations. On the right, it is acting on oversaturated solutions containing an increasing number of emulsified particles.

It is seen that the presence of NaCl induces a relatively high lipase activity in the isotropic solutions. The value at the saturation point is about 30% of the maximal activity observed later for emulsions of triacetin and 17% for tripropionin. In contrast to our previous assays in the absence of NaCl (ref. 1), there is no abrupt discontinuity at the saturation point. On the other hand, the curves for the isotropic solutions are clearly biphasic. For dilute solutions, the rate is low, and it increases slowly with the concentration. Beyond an apparently critical concentration, the activity increases

much faster. The significance of this critical concentration will be discussed later.

The curves for the emulsions are, as pointed out earlier, Michaelis curves describing the progressive adsorption of lipase at the interface of the particles. A maximal rate is finally attained when all enzyme molecules are adsorbed. It is confirmed that this rate is strongly affected by the chain length of the substrate[12,13]. Under the conditions used here, a 20-fold variation is observed between tripropionin (3-carbon chains) and triacetin (2-carbon chains).

Results obtained with dibutyrin (Fig. 3) are essentially the same as for triacetin and tripropionin except that the critical concentration is less apparent. This is probably due to the concentration scale which is smaller here than for tripropionin. If the critical concentrations for dibutyrin and tripropionin are of the same order, the first one cannot be seen in the diagram of Fig. 3. It may be added that the rate observed just below the saturation of dibutyrin solutions is 40% of the maximal value obtained later with dibutyrin emulsions. This maximal value is 17-fold lower than for tripropionin. Since tripropionin is known to be hydrolyzed by lipase at about the same rate as tributyrin[12,13] it can be concluded that the passage from tributyrin to the corresponding 1,3-diglyceride slows down the catalytic process by a factor of about 17.



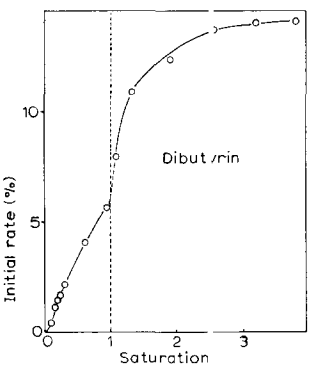


Fig. 2. Hydrolysis rate of triacetin and tripropionin as a function of substrate concentration. Initial rates were measured in a pH-stat as indicated in the text. They are expressed in per cent of the maximal rate observed with the same amount of lipase and an excess of a triolein emulsion. The total concentration of the substrate (solution + emulsion) is expressed in multiples of the saturation. The vertical dashed line indicates the saturation point.

Fig. 3. Hydrolysis rate of dibutyrin as a function of substrate concentration. Same legend as for Fig. 2.

Relationship between lipase activity on concentrated solutions and aggregation of the substrate

The shape of the curves obtained on the left of the saturation point in Fig. 2 suggests that lipase does not act indiscriminately on all substrate molecules in the isotropic solution, but rather on some functional units that would accumulate when the concentration of the solutions is high. According to Fig. 1, the formation of these units are favored by NaCl. Since NaCl is known to lower the critical micellar concentration of various amphipaths[9], a likely hypothesis is that the units mentioned above are molecular aggregates. In other words, there would be no abrupt transition between the fully dispersed glyceride solutions and the emulsions. Short chain glycerides would

begin to associate in the presence of 0.1 M NaCl well before the solutions were saturated.

Preliminary experiments have shown that tripropionin aggregates can be detected by light scattering in concentrated solutions containing NaCl. A series of assays using the dye techniques mentioned in the preceding section are described below. These techniques, currently employed to determine the critical micellar concentration of amphipaths, make use as stated earlier of the solubilization effect for water-insoluble Sudan III or of the spectral shifts for benzopurpurin and iodine that occur when the dyes are incorporated into micelles. The results obtained with triacetin and tripropionin solutions of varied concentrations are given in Fig. 4.

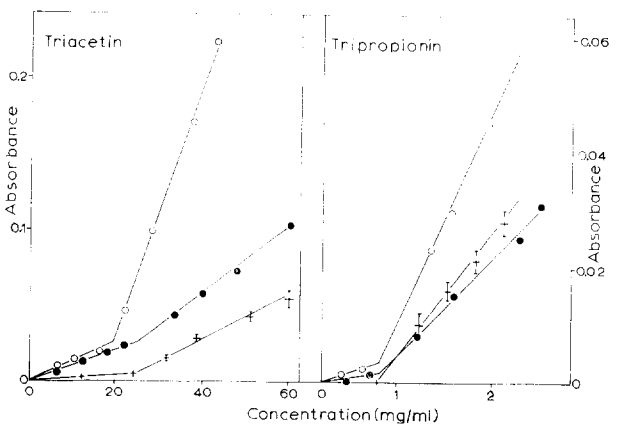


Fig. 4. Detection of molecular aggregates in triacetin and tripropionin solutions. ○ and ●, differential absorbance read at 510 and 355 mμ, respectively, for benzopurpurin 4 B and iodine. Crosses, solubilization of Sudan III measured by the absorbance of the solutions at 505 mμ. For this latter determination, the standard errors are indicated by the vertical part of the crosses. All assays were carried out at 25°.

The curves of Fig. 4 resemble those obtained with micelle-forming amphipathic compounds. They are composed of two linear parts with different slopes. The abscissae of the intercept can therefore be assumed to indicate a critical value of the concentration, perhaps similar to the critical micellar concentration of amphipaths, above which triacetin or tripropionin molecules associate at 25° in the presence of 0.1 M NaCl. These values are about 23 mg/ml for triacetin and 0.7 mg/ml for tripropionin. Under the same conditions, the concentrations corresponding to the saturation of the solutions are 72 and 2.5 mg/ml, respectively. It may be postulated that, below the critical concentration, most glyceride molecules are fully dispersed in water. Between critical concentration and saturation, these molecules associate to give aggregates in an isotropic solution. Above the saturation, the size of the aggregates suddenly increases to form large particles in a turbid emulsion.

An additional indication that short chain glycerides really associate in our system is given by the observation that the maximal spectral shift for benzopurpurin and iodine corresponds to nearly the same wavelength as for ordinary amphipaths (510 and 355 mμ instead of 530 and 350 mμ). Similar experiments with dibutyrin have not yet been carried out.

It is of interest to know whether a correlation can be established between lipase

activity in isotropic solutions and the concentration of the aggregates in these solutions. In Fig. 5 the two series of parameters are plotted for each concentration in per cent of their maximal values at the saturation point. The responses of the techniques used for the detection of aggregates closely follow the activity curve. In very dilute solutions below the critical value, the aggregate concentration and the hydrolysis rate are low. Both increase slowly when the total concentration of the substrate is raised. Above the critical value, they increase much faster in a parallel way. This indicates that, in contrast with earlier belief, lipase is able to act, not only on emulsified particles of the substrate, but also on much smaller aggregates in an isotropic system. Moreover, the experimental observations discussed above are consistent with the view that the activity of lipase on fully dispersed substrate molecules is considerably lower than on aggregates and particles. But it cannot yet be said with certainty that this activity does not exist. The weak activity observed below the critical concentration may at least partly be attributed to the presence of some aggregates already found in this range. However, if lipase were exclusively acting on the aggregates, the tangent of the curve for a concentration 0 should be horizontal. This is obviously not so for triacetin. Thus, it is possible, but by no means certain, that lipase has a slight activity on dispersed molecules.

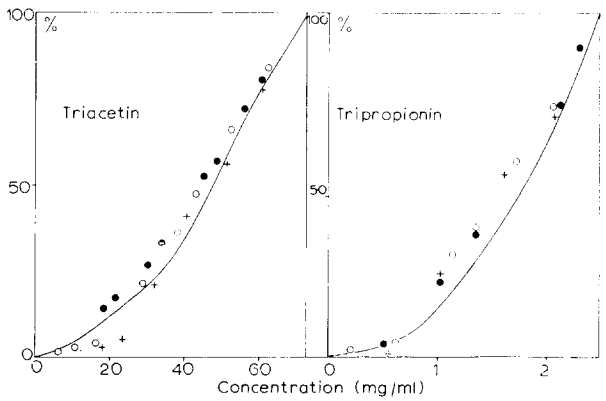


Fig. 5. Correlation between lipase activity and the formation of aggregates in triacetin and tripropionin solutions. The continuous curve indicates lipase activity *versus* substrate concentration. The other signs indicate the responses of the techniques used for the detection of aggregates. The signs are the same as in Fig. 4, namely white circles for benzopurpurin, black circles for iodine and crosses for Sudan III. The ordinates are expressed in per cent of their maximal values at the saturation point.

Nature of the enzyme acting on aggregates

It has been assumed so far that lipase itself catalyses the hydrolysis of aggregated glyceride molecules. However, the preparations used here were not quite pure, and MORGAN *et al.*[4] recently pointed out that mixed micelles of 1-mono-olein and taurodeoxycholate are attacked by an enzyme in rat pancreatic juice. This enzyme has an apparent molecular weight of about 70 000, and it can be separated from lipase by a filtration through Sephadex G-100. It is therefore desirable to prove unambiguously that the same enzyme, namely lipase, hydrolyzes emulsified particles as well as smaller aggregates of the same substrate.

Accordingly, isotropic solutions of 1-mono-olein–taurodeoxycholate micelles

were prepared as described by MORGAN *et al.*[4] and were incubated for 60 min at 25° and pH 5.8 with purified porcine lipase or with a fresh sample of rat pancreatic juice. As indicated by the authors, the liberated fatty acids were extracted and titrated. This activity was compared with the normal activity of lipase on a triolein emulsion[6]. The number of lipase units was the same for each of the two experiments serving for comparison. The results are listed in Table I.

TABLE I

RELATIVE ACTIVITY OF PORCINE LIPASE AND RAT PANCREATIC JUICE ON 1-MONO-OLEIN-BILE SALT MICELLES AND ON A TRIOLEIN EMULSION

All experiments were performed with the same lipase preparation. The figures indicate the number of enzyme units used in each case.

Source of enzyme	*Lipase activity (μmoles free fatty acids per min)*		*Ratio (%) of activities against micelles and emulsions*
	On a triolein emulsion	*On 1-mono-olein bile salt micelles*	
Rat pancreatic juice	9.5	0.17	1.7
	28.5	0.56	1.9
Purified porcine lipase	9.0	0.00	0.00
	22.5	0.013	0.05
	90.0	0.16	0.17
	225.0	0.15	0.06

Table I shows that, in agreement with the findings of MORGAN *et al.*[4], rat pancreatic juice displays on the mixed micelles a detectable activity (about 2% of the maximal activity of the enzyme on a triolein emulsion). But this activity is much lower or even nonexistent in our purified porcine lipase preparations. Consequently, the triacetin and tripropionin aggregates investigated here and the mixed micelles are certainly not hydrolyzed by the same enzyme. Since lipase is inhibited by low concentrations of bile salts, it would hardly be expected to act on micelles containing large amounts of these compounds.

In order to prove that triacetin and tripropionin aggregates are actually hydrolyzed by lipase, 1 ml of a solution (6200 lipase units; specific activity, 3000) of the purified lipase preparation in 0.15 M NaCl was filtered through a Sephadex G-100 column. The column (0.9 cm × 145 cm) was equilibrated with, and eluted by, 0.15 M NaCl. The activity of each fraction (2 ml) was measured at pH 9 on a triolein emulsion (lipase activity) and at pH 7 on a 0.8 saturated triacetin solution or a 0.5 saturated tripropionin solution in 0.1 M NaCl. Fig. 6 shows that the three activities emerge together from the column. There is no indication of any activity in the region of the chromatogram where proteins of 70 000 molecular weight are normally eluted. The conclusion is that triacetin and tripropionin aggregates are hydrolyzed by lipase.

The chromatography described above eliminates from the lipase peak some faster- and slower-migrating inactive compounds. After chromotography, the prepa-

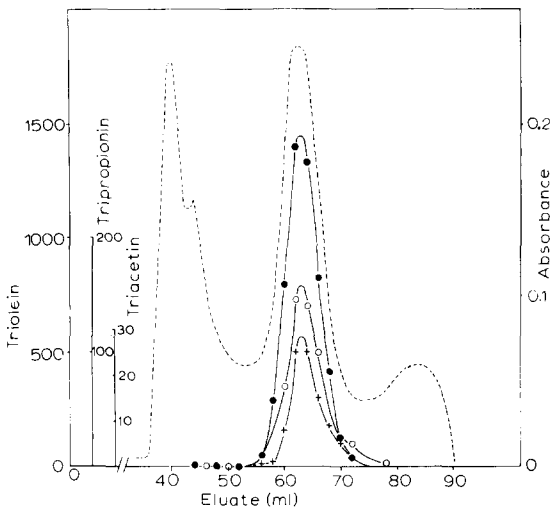


Fig. 6. Chromatography of porcine lipase on Sephadex G-100. The conditions of the chromatography are described in the text. The substrates on which the activities of the fractions are measured are a triolein emulsion (black circles) and a 0.8 saturated triacetin or a 0.5 saturated tripropionin solution (crosses or white circles, respectively). The dashed line gives the absorbance of the fractions at 280 mμ (total proteins of the fractions).

ration is completely free of any impurities detectable by disc electrophoresis. This is further confirmation that lipase itself is acting on aggregates.

Hydrolysis rates with aggregates and emulsified particles

A very large difference in size exists between aggregates in an isotropic solution and emulsified particles. It would therefore be of interest to compare the kinetic parameters (K_m and v_{max}) of lipase for the same substrate taken successively in these two physical states. Whereas a comparison of the K_m is presently difficult, the v_{max} can readily be compared on the basis of existing data.

In the isotropic solutions, the concentration of the most functional substrate, namely the aggregates, can be obtained by subtracting the critical concentration from the total concentration. The concentration of the emulsified substrate in the emulsion is the difference between total concentration and concentration at the saturation point. As far as the activities are concerned, the activity of lipase on isotropic solutions can be assumed to be entirely directed against aggregates. The case of emulsions appears at first sight to be more complicated since the activity measured in this region is the sum of the effect of lipase on aggregates and on emulsified particles. A further complication arises from the competition that must occur between aggregates and emulsified particles for the binding of lipase, so that the amount of enzyme acting on emulsified particles probably varies with the concentration of the emulsion. However, the error made when the total activity in emulsion is attributed to the hydrolysis of emulsified particles becomes negligible for concentrated emulsions in which lipase is almost entirely bound to the particles. Hence, the last experimental points on the left of the emulsion plots in the Lineweaver–Burk representations (Figs. 7 and 8), and consequently the extrapolations to infinite concentrations giving the v_{max} values for emulsions, are likely to be correct.

Figs. 7 and 8 give the Lineweaver–Burk representations for the action of lipase on aggregates and emulsified particles of triacetin and tripropionin. The position of the points has been calculated according to the assumption discussed above. The abscissae are the reciprocal values of the total substrate concentrations minus the critical concentration for the solutions or minus the saturation for the emulsions. The ordinates are the reciprocal values of the total activities measured in the solutions and the emulsions, respectively.

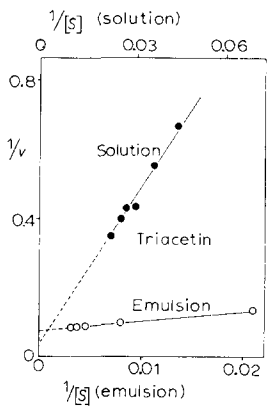


Fig. 7. Lineweaver–Burk plots for triacetin solutions and emulsions.

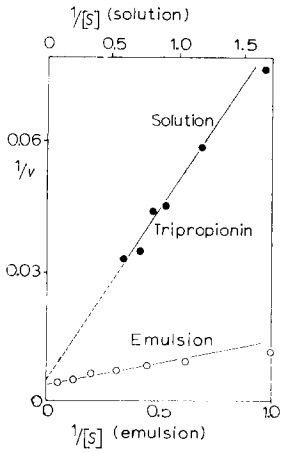


Fig. 8. Lineweaver–Burk plots for tripropionin solutions and emulsions.

For both substrates, the extrapolation to infinite concentration of the emulsion and aggregate plots are very close to each other. It appears therefore, within the relatively high experimental error associated with this kind of study, that the maximal rate of lipolysis of the same substrate in an aggregated and an emulsified form is similar, if not identical. This result suggests that the primary enzyme–substrate complex once being formed, the subsequent catalysis proceeds in the same way for aggregates and particles. Lipase would act on aggregates at a very high rate were it possible to raise their concentration well above the corresponding K_m. However, this cannot be done in practice, at least under the conditions and with the substrates employed here, because of the saturation of the solutions.

The example of 1,3-dibutyrin will be discussed in more detail when the critical concentration of this compound is known. However, it is interesting that, if this critical concentration is in the same range as for tripropionin, the maximal hydrolysis rate for dibutyrin aggregates and particles will also be similar.

As mentioned earlier, preliminary experiments have been carried out to investigate more closely by light scattering the association of tripropionin molecules in 0.1 M NaCl. From the few results already available, these aggregates appear to be of a rather uniform size and to contain about 15 monomers. Triacetin aggregates are much smaller, so that an evaluation of their size by light scattering is difficult. It would be interesting to know why and how the state of aggregation of the substrate so strongly influences lipase action.

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