J. Lipid Research, January, 1962
Volume 3, Number 1

The acid lipase of the castor bean. Properties and substrate specificity*

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[Received for publication June 15, 1960]

SUMMARY

A castor bean lipase with a pH optimum of 4.3 is found within the fatty layer obtained by centrifuging a homogenate of the bean; most of the fat may be removed by extraction with ethyl ether in the presence of saturated sodium chloride solution, yielding a preparation which consists of about 55% protein and 5% lipid and which is insoluble in neutral buffer. This preparation emulsifies triglycerides and hydrolyzes them completely; the enzyme is inhibited by mercurials but not by diisopropylfluorophosphate. The data on effect of enzyme concentration on hydrolysis rate suggest that a second component, perhaps an emulsifier, is required for activity. Hydrolysis of triglycerides of saturated fatty acids is maximum at a chain length between C_4 and C_8. Triacetin is not hydrolyzed. Glycerides of long-chain fatty acids, predominantly unsaturated C_{18}, are also hydrolyzed, but not as rapidly as the maximum for the glycerides of short-chain fatty acids. Mono- and dibutyrin are hydrolyzed less rapidly than tributyrin, but diolein is hydrolyzed more rapidly than triolein; this suggests that there may be two separate enzymes for the short-chain and long-chain triglycerides, respectively. Esters of ricinoleic acid and monohydric alcohols are hydrolyzed at a much lower rate than is castor oil.

The action of lipase on triglycerides of long-chain fatty acids requires that the substrate be emulsified. A large variety of emulsifiers has been employed including: gelatin; gum arabic (1); sodium taurocholate (2); and a mixture containing the fatty acid ester of polyethylene glycol, soybean phosphatides, sorbitan monolaurate, sodium cholate, and a polyglycerol ester of fatty acids (3). By such means the substrate specificity of lipase from various sources on water-insoluble substrates has been measured. But it has been shown that the substrate specificity of a lipase is influenced by the nature of the emulsifying system (4). The question arises, therefore, as to the existence of natural emulsifiers for individual lipases; investigation of this combination would provide more information on the variables controlling substrate specificity.

Oleaginous seeds might be considered good material for study of natural emulsifiers, since no exogenous sources of emulsifiers participate in the mobilization of fat. In oleaginous seeds, lipase activity is manifest upon germination, but in the castor bean (*Ricinus communis*), there is also an active lipase in the resting seed. This, therefore, provides a convenient source for study of both the enzyme and possible emulsifiers.

Crude preparations from the castor bean have been reported to catalyze the hydrolysis of a wide variety of esters. These include: glycerides of long-chain fatty acids (5, 6, 7); triacetin and tributyrin (7, 8); various esters including the butyl and amyl esters of formic, propionic, and butyric acids (9, 10); and methyl mandelate (11). In most of the work that has been reported, oil-free kernels served as the enzyme (5, 12, 13). Bamann *et al.* (14) described attempts to solubilize the lipase from the castor bean; only 20% was soluble in dilute ammonia. This is the well-known *Ricinus* lipase first described by Green (15). Obviously, the crude preparations had the properties of an ali-esterase, in addition to being a lipase.

We have reported previously on a castor bean lipid concentrate, which contains the enzyme capable of hydrolyzing the endogenous lipid substrate (16). The lipase is extracted, together with its substrate, as a fatty layer by macerating water-swollen castor beans in pH 7 buffer and centrifuging; the fatty layer contains both the enzyme and substrate. This procedure has the advantage of eliminating most of the soluble proteins of the castor bean from the enzyme prepara-

* Presented in part at the meeting of the Federation of Biological Societies, Atlantic City, April 13–17, 1959.

tion. Since castor beans contain allergens, which have interfered with the progress of research on this enzyme, the elimination at the first stage of the majority of the proteins and allergens obviates this difficulty.

This lipid concentrate, containing only about 5% of the original nitrogen of the kernels, is further purified by removal of lipids and some proteins to yield an insoluble preparation capable of hydrolyzing water-insoluble fats without additional emulsifiers (17). This preparation may be dialyzed and lyophilized, and is stable for several months at refrigerated temperatures.

This report deals with the properties of this lipase preparation and its substrate specificity. The preparation has a broad range of activity on triglycerides from tripropionin to glycerides of long-chain fatty acids, limited activity on esters of monohydric alcohols, and no activity on triacetin.

EXPERIMENTAL METHODS

Measurement of Activity. Two methods were employed to measure lipase activity: in one, the rate of decrease in ester content was measured; and in the other, the increase in titratable acidity. The first was based on the determination of unhydrolyzed ester as the ferric hydroxamate colored complex. Enzyme, substrate (ca. 500 μmoles of ester bond), and water (to a volume of 6 ml) were emulsified at 25° for 5 minutes in a Potter-Elvehjem homogenizer. Acetate buffer, 0.25 M (4 ml), was added to adjust to pH 4.2, and aliquots (0.01 to 0.1 ml) were withdrawn at 5-minute intervals. Normally a reaction period of 20 minutes, with constant stirring by a magnetic stirrer, was adequate. The aliquots were boiled, filtered, and reacted with alkaline hydroxylamine as described previously (16). The ferric hydroxamate colored complex was read at 520 mμ with refined cottonseed oil as a standard for calibration. A minimum change of 0.3 μmole of ester could be detected.

The initial rate of hydrolysis was extrapolated from the plot of ester content versus reaction time. At the substrate concentration for many of the data reported (100 to 200 μmoles of ester bond per ml of reaction mixture), the rate of the reaction was pseudo-first order with respect to the substrate concentration. In those instances the initial rate of hydrolysis was calculated by multiplying the rate constant (decrease in logarithm of ester concentration per unit time multiplied by 2.3) by the initial ester concentration. The choice of lower levels of substrate concentration was dictated in some instances by a desire to reproduce that concentration of substrate found in the crude enzyme preparation in the fatty layer (16). This procedure was time-consuming and subject to sampling errors; hence it was replaced by the second procedure used for most of the data reported herein. For those data reported by the first method, the reproducibility is within 5%.

In the second procedure, 2.5 mg of enzyme (ca. 55% protein) was homogenized with 0.1 ml of 0.1 M Tris[1] buffer, pH 8.0, the required amount of substrate, and 1.4 ml of water. This was poured into a reaction vessel and rinsed with water to a final volume of 4.9 ml. Then the stirring was begun, pH was measured, and 0.05 to 0.1 ml of 0.1 N acetic acid was added to lower the pH to 4.2, starting the reaction. During the reaction, the pH was maintained constant. After 10 minutes at 25° or 40°, 5 ml of absolute ethanol was added and the mixture titrated automatically with 0.1 N sodium hydroxide to pH 8.5, with a Radiometer pH-stat titrimeter.[2] At high substrate concentrations, the rate of formation of fatty acid was pseudo-zero order with respect to substrate concentration. The reproducibility by this method was within 3%. Substrate concentration is expressed in terms of moles of ester bond per milliliter.

No additional emulsifier was needed for activity; in the presence of the enzyme, the substrates remained emulsified for several hours. There was no need for fatty acid acceptors, such as calcium ion or serum albumin.

Seed. Castor beans of the Cimmaron and Baker 296 varieties were obtained through the courtesy of D. S. Bolley and W. E. Domingo of the Baker Castor Oil Company. No observable difference was found in the yield of lipase between the two varieties nor in storage of the seed up to 2 years at 15°.

Purification of Enzyme. A substantial purification of the acid lipase is accomplished simply by centrifuging the extract of the ground castor bean; the enzyme is found in the fatty layer, whereas the great bulk of the protein remains in the infranatant solution and the residual debris. The enzyme is purified further by removal of fat and some protein, leaving a preparation which, though insoluble, contains no endogenous substrate and therefore can be tested for substrate specificity.

The fatty layer containing both enzyme and substrate was obtained by extracting the water-swollen seed with 0.1 M phosphate buffer at pH 7 containing, additionally, 0.05 M cysteine and 0.01 M EDTA, and centrifuging. It was transferred by spatula to a glass-stoppered graduated cylinder, an equal volume of satu-

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; PCMB, p-chloromercuribenzoic acid; DFP, diisopropylfluorophosphate; Tris, tris(hydroxymethyl)aminomethane.

[2] Use of a trade name does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

rated sodium chloride containing 0.05 M cysteine at pH 7 was added, and the remainder of the space in the vessel was filled with ether. Contents were mixed gently by means of an enclosed glass marble and allowed to stand at 4° for 1 hour. Then the mixture was centrifuged at low speed to form three phases: the upper layer containing the extracted lipid, the middle layer containing the enzyme, and the lower layer containing salt-soluble material. Both the upper and lower phases were removed by syringe; the solid layer remaining in the graduated cylinder was re-extracted twice more with both ether and saturated salt and then diluted with 2 volumes of the phosphate buffer containing cysteine and EDTA. This was homogenized in a Potter-Elvehjem glass homogenizer, dialyzed against running distilled water, then lyophilized. The final preparations contained 55% to 60% protein; in one example, this represented 2.7% of the original nitrogen and 0.06% of the original lipids.

It was interesting to compare the amount of activity recovered in the purified enzyme to that originally present in the fatty layer. Even though the test was done by exactly the same technique (decrease in ester content), and with the substrate (castor oil) adjusted to the same concentration in both instances (137 μmoles of ester per ml), there is no way of knowing from present information whether the substrate in the fatty layer and the exogenous castor-oil substrate are exactly the same or whether there are other components of the endogenous system not yet identified which are not present, or present in lesser concentration, in the purified system. In one experiment, 60% of the activity present in the fatty layer was recovered; the specific activity of the enzyme (μmoles of ester hydrolyzed per mg protein per minute) was 5.1 in the fatty layer and 7.9 in the purified material.

The enzyme in its present form is insoluble in aqueous media and is probably attached to particulate matter. The mildest possible conditions for removal of oil were chosen and oil removal was conducted at low temperature to avoid denaturation.

Substrates. Most of the substrates were not pre-emulsified prior to testing; hence, any emulsification required for activity depended on the presence of such properties in the enzyme preparation. In some instances, for comparison, cottonseed oil was pre-emulsified with commercial emulsifiers.

Cottonseed oil was a commercial preparation of refined and bleached oil (Wesson Oil). Castor oil was Baker Castor Oil Company, DB grade refined. The cottonseed-oil emulsion was obtained through the courtesy of W. S. Singleton and was similar to that described by Singleton *et al.* (18) except that it contained

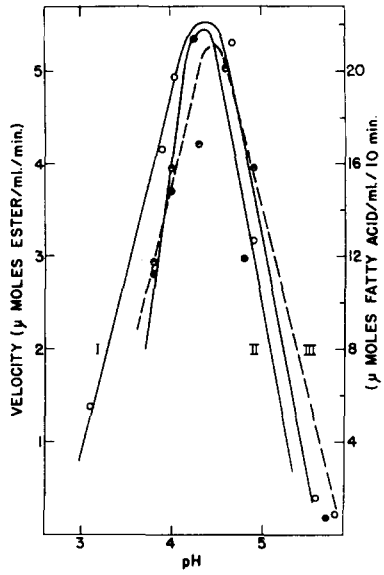

FIG. 1. Effect of pH and temperature on activity of castor acid lipase. I: 25° cottonseed-oil emulsion; 500 μmole ester, 10 mg protein (N × 6.25) in 10 ml reaction volume; decrease in ester content was measured colorimetrically. II and III: Temperatures were 25° and 40°, respectively; cottonseed oil; 1000 μmole ester and 1.4 mg protein in 5 ml reaction volume; increase in fatty acids titrated with 0.1 N NaOH.

no dextrose. Methyl and butyl ricinoleate and a partially purified egg lecithin were furnished through the courtesy of D. A. Yeadon and H. P. Dupuy. Myverols, Type 18-17 glycerol monooleate, Type 18-98 monoglycerides of safflower oil, and diolein were gifts of R. F. Liming and Morris Embree of Distillation Products Industries. Triacetin; tri-, di-, and monobutyrin; tripropionin; tricaprylin; tricaprin; trilaurin; trilinolein; and triolein were commercial preparations further purified by passage over an alumina column.

RESULTS

Stability. The purified lipase was stored for 60 days in the cold as a suspension without loss of activity. The suspension was kept in the same medium used for extracting the enzyme from the kernels—35 mg of the enzyme per ml of phosphate buffer, pH 7.0, containing cysteine and EDTA. Another portion of this suspension was lyophilized and stored at 4°; a third was dialyzed against distilled water, lyophilized, and stored at 4°. These were analyzed 6 times during a 60-day storage period; the average lipase activity for this period with 1 mg of protein per ml and cottonseed-oil emulsion as the substrate (50 μmole per ml) was 4.1 μmole ester hydrolyzed per mg protein per minute for the two lyophilized preparations and 5.0 μmole ester per mg per minute for the suspension. Similar stability

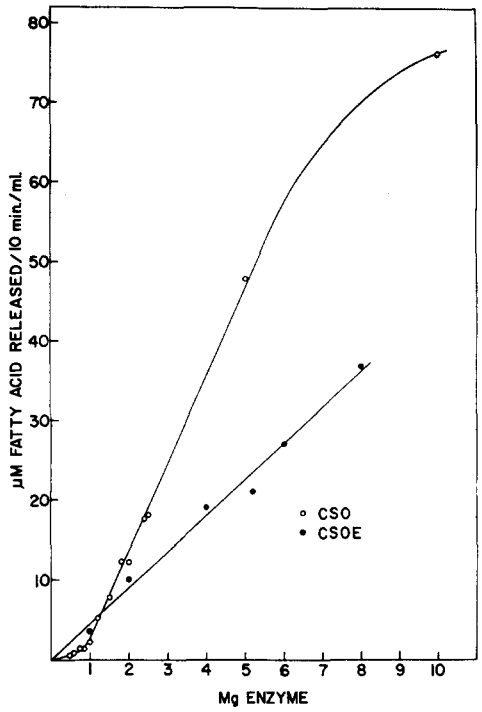

FIG. 2. Effect of enzyme concentration on activity of castor lipase. Each flask contained 1,000 µmole substrate ester, 0.1 ml Tris buffer pH 8.0, enzyme (55% protein), 0.05 ml 0.1 M acetic acid, and water to 5 ml; temperature 25°, reaction time 10 minutes at pH 4.25; CSO, cottonseed oil; CSOE, cottonseed-oil emulsion; hydrolysis measured by titration of fatty acids with 0.1 N NaOH.

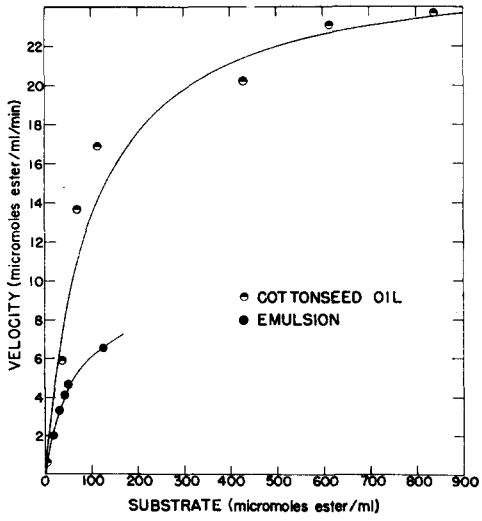

FIG. 3. Effect of concentration of cottonseed oil and cottonseed-oil emulsion on the activity of castor lipase at 25°. Each flask contained 10.6 mg protein plus substrate in a total volume of 10 ml. Decrease in ester content was measured colorimetrically.

was exhibited when unemulsified oils were employed as substrate. Other lipase preparations, dialyzed and lyophilized, have been stored in the frozen state for periods of 5 months without a loss in activity.

pH Optimum. Figure 1 gives the effect of pH on the initial rate of hydrolysis. There is slight, if any, difference in the pH optimum (about 4.3) of the oil or the emulsion at 25° by either method of analysis, nor is there a significant change in the pH optimum with increase in temperature. Likewise, there is no appreciable difference in the rate of hydrolysis at the two temperatures tested (II and III, Fig. 1).

Effect of Enzyme Concentration. The effect of concentration of enzyme on the initial rate of hydrolysis of cottonseed oil and a cottonseed-oil emulsion is shown in Figure 2. The curve for cottonseed oil is sigmoid; there is a linear portion extending over a wide range of enzyme concentration. The conditions for measuring enzyme activity in other experiments fell within this linear portion. The curve for the effect of enzyme concentration on hydrolysis of the cottonseed-oil emulsion is linear and passes through the origin.

Mercuric Chloride Inhibition. The lipolysis of endogenous substrate in the castor bean is inhibited by mercuric ion and PCMB; this inhibition is reversed by incubation with cysteine and EDTA (16). Similarly, the activity of the purified lipase on a cottonseed oil emulsion was inhibited completely by 5×10^{-4} M $HgCl_2$. This inhibition was reversed partially (70%) by incubation in pH 7 phosphate buffer containing 0.05 M cysteine and 0.01 M EDTA. Sodium thioglycolate and dithiopropanol (0.5 M) were less effective (40% recovery), and glutathione (0.05 M) was ineffective in reversing the inhibition.

Diisopropylfluorophosphate. This reagent did not inhibit castor lipase activity on the endogenous substrate except at relatively high concentrations (16), but the possibility remained that the enzyme in that instance was being protected by the substrate. Incubation of the purified lipase with DFP(10^{-3} M) for 1 hour, either at pH 7 or at pH 4, did not inhibit hydrolysis; actually there was some stimulation of activity (125% of untreated enzyme).

Effect of Substrate Concentration. Curves typical of the effect of increasing the substrate concentration on rate of hydrolysis at 25° are shown in Figure 3. At comparable substrate concentrations, cottonseed oil is hydrolyzed more rapidly than the cottonseed-oil emulsion. Maximum rate for cottonseed oil requires relatively high substrate concentrations; for tributyrin, the maximum rate is attained at a concentration of 200 µmole ester per ml.

At 40° the rates of hydrolysis of the butyrins de-

TABLE 1. HYDROLYSIS OF VARIOUS SUBSTRATES BY CASTOR ACID LIPASE*

Substrate	Fatty Acid Produced	
	25°	40°
	μmole/10 min/ml	*μmole/10 min/ml*
Castor oil	21.1	27.6
	25.9†	
Butyl ricinoleate	3.7	3.1
Cottonseed oil	21.4	20.2
	25.5†	
Triolein	18.7	21.2
Diolein	36.2	48.5
Trilinolein	22.0	23.9
Tributyrin	47.2	40.0
Dibutyrin	28.7	23.0
Monobutyrin	10.4	9.0

* Each flask contained 1000 μmole of substrate ester, 2.5 mg of enzyme, 0.1 ml Tris buffer pH 8, 0.1 ml 0.1 M acetic acid, and water to 5 ml. Reaction time was 10 minutes; hydrolysis was measured by titration of the fatty acids produced with 0.1 N NaOH.

† Contained 2000 μmole of substrate ester.

crease from the maximum at higher substrate concentrations. This is shown in Figure 4 for hydrolysis of tri-, di-, and monobutyrin. Since the data are plotted on the basis of ester content, it is not surprising that the maxima are at about the same concentration. The equivalents of ester bond, and not molecules of glyceride, control the rate of hydrolysis.

The amount of ester emulsified per ml is not in itself the substrate concentration; the substrate is not in solution. Only the portion on the active surface would be reacting. Therefore, substrate concentration has only comparative meaning.

In general, it would seem that at the lower temperature (25°) there is less tendency for a fall-off in rate from the maximum at high substrate concentration. Because the rate of hydrolysis at 200 μmoles of substrate ester per ml is close to the maximum, all subsequent measurements on the effect of substrate type were made at that concentration.

Effect of Substrate Type.

(*a*) *Emulsion.* The hydrolysis of cottonseed oil is more rapid than that of the same oil emulsified with commercial emulsifiers prior to contact with the enzyme (Fig. 3).

(*b*) *Effect of Chain Length of Fatty Acids in Triglycerides.* The effect of chain length on hydrolysis rate for a series of triglycerides of saturated fatty acids from triacetin to trilaurin is shown in Figure 5. Maximum activity lies between C_4 and C_8 fatty acids in the triglyceride. It is interesting that no activity is shown for triacetin. We have examined triacetin as a sub-

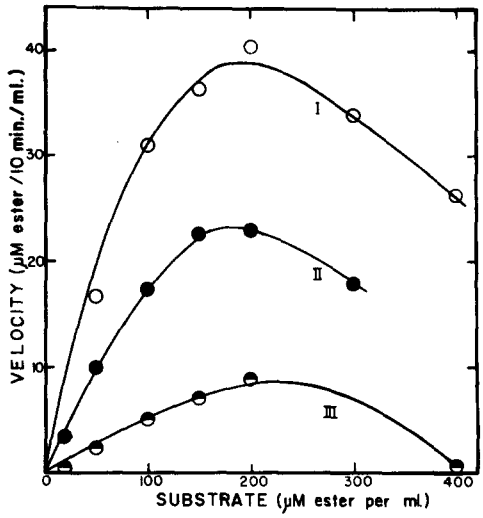

FIG. 4. Hydrolysis of butyrins by castor lipase at 40°. Each flask contained 1000 μmole ester, 0.1 ml Tris buffer pH 8.0, 1.4 mg protein, 0.05 ml 0.1 M acetic acid, and water to 5 ml; reaction time, 10 minutes at pH 4.5; hydrolysis measured by titrating fatty acids with 0.1 N NaOH. I: tributyrin; II: dibutyrin; III: monobutyrin.

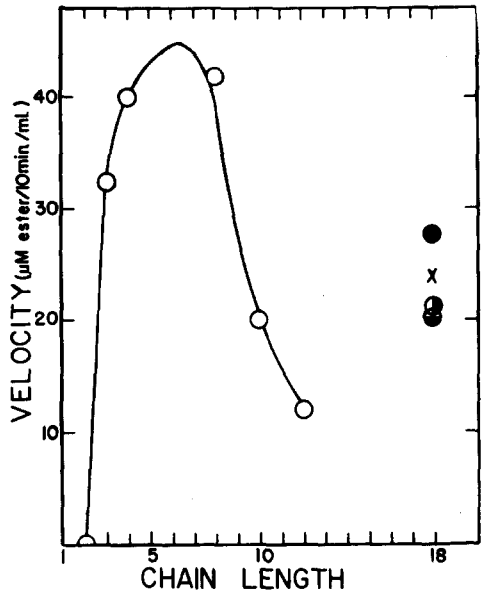

FIG. 5. Effect of fatty acid chain length on hydrolysis of triglycerides by castor lipase at 40°. Each flask contained 1.4 mg protein, 0.1 ml Tris buffer, pH 8.0, 1,000 μmole ester, 0.05 ml 0.1 M acetic acid, and water to 5 ml; reaction time, 10 minutes at pH 4.5; hydrolysis measured by titrating fatty acids with 0.1 N NaOH. ●, castor oil; ×, trilinolein; ◑, triolein; ◒, cottonseed oil.

strate over a range of concentrations up to 2,000 μmoles of ester per ml; in all instances there was no activity.

Four oils containing predominantly C_{18} fatty acids are hydrolyzed at about the same rate, but lower than the maximum rate attained with triglycerides of shorter chain saturated fatty acids, as shown in Table 1. Even at concentrations of 400 μmoles per ml, which approach maximum rate (see data of Fig. 3 for cottonseed oil obtained on another enzyme preparation and therefore not quantitatively comparable), cottonseed and castor oils are hydrolyzed at a rate substantially lower than the shorter chain triglycerides. All of these oils are hydrolyzed at a higher rate than trilaurin; but since they are composed primarily of unsaturated fatty acids, they cannot be compared directly to the saturated fatty acids. Increase in degree of unsaturation from triolein to trilinolein provides a slight increase in the rate of hydrolysis.

(*c*) *Tri-, Di-, and Monoglycerides.* In Figure 4 are compared the relative rates of hydrolysis of tri-, di-, and monobutyrin at 40°. Tri- and diolein are compared in Table 1. It was not possible to measure the rate of hydrolysis of monoolein at comparable concentration because this would require additional emulsifiers and would confound interpretation of the results. It is clear that in tri-, di-, and monobutyrin, successive removal of one fatty acid from the glyceride decreases the rate of hydrolysis of the remaining esters. This is not true for the series triolein and diolein; there is a sharp increase in the rate of hydrolysis of diolein as compared to triolein.

(*d*) *Other Esters.* Butyl ricinoleate is compared to the other substrates at the same ester concentration in Table 1. Obviously it is hydrolyzed at a much lower rate than the corresponding triglyceride, castor oil. In other experiments, methyl ricinoleate was found to be hydrolyzed at about the same rate as butyl ricinoleate. Lecithin was not hydrolyzed at all by this enzyme preparation.

Effect of Temperature. There is no uniform effect of temperature on the rate of hydrolysis of the substrates studied. For most of the substrates, the rate of hydrolysis is unaffected by temperature, but there are some exceptions. The butyrins are hydrolyzed less rapidly at the higher temperature than at the lower temperature, while castor oil and diolein are hydrolyzed more rapidly at the higher temperatures. Interpretation of the temperature effects is not simple, since temperature affects not only the rate of the hydrolytic reaction but also the nature of the surface. In the case of castor oil and diolein, there is a possibility that their viscosity, which is reduced at the higher temperature, is an important factor.

DISCUSSION

The sigmoid-shaped curve in Figure 2, obtained when measuring the effect of increasing the enzyme concentration with cottonseed oil as the substrate, deserves further comment. The same shaped curve was noted by Mattson and Beck (19) with pancreatic lipase, and a similar shaped curve was noted by Koch *et al.* (20) in studies of soybean lipoxidase, particularly on purified preparations. The latter authors ascribed this shape to the induction period observable in the first few minutes of reaction. However, the sigmoid curve was not observed with impure enzyme preparations. A more likely explanation, for the data reported here at least, is that a second component is necessary for activity, perhaps an emulsifier (21). This possibility is being further investigated. Further support for this point of view comes from the fact that, with an emulsion as the substrate, the curve is linear and passes through the origin.

This second component may be responsible for the emulsifying action of the enzyme; or this material might be similar in nature to the class of lipids such as cardiolipin, inositol phosphatides, or certain synthetic emulsifiers required for activity of some phospholipases (22, 23). In this instance, these are not necessarily emulsifiers, but they make it possible for the enzyme to combine with the substrate under conditions of substrate surface concentration such as would be encountered in emulsified substrate globules.

For glycerides of saturated fatty acids, maximum activity is at a chain length between C_4 and C_8. Lipmann and Tuttle (24) found a maximum for hydroxamic acid formation at C_8 for hog liver extract and at C_{12} for pancreatic lipase. Lynn and Perryman (4) found maximum activity for adipose tissue lipase with tributyrin as the substrate. It is interesting to note that this castor bean acid lipase has no activity on triacetin, regardless of concentration of the substrate. In this respect, it differs from pancreatic lipase (25) but does not differ from adipose tissue lipase (4).

The mono- and diglycerides of butyric acid are hydrolyzed successively less rapidly than the triglyceride; this is similar to the behavior noted with pancreatic lipase on oleins (3) and other long-chain triglycerides (2). The fact that diolein is hydrolyzed much more rapidly by the castor bean enzyme than triolein might lead one to suspect that there are at least two enzymes in this mixture: one hydrolyzing the glycerides of the short-chain fatty acids and the other the glycerides of the longer chain fatty acids. The pattern for tri- and diolein is more typical of the published information on castor bean lipase (12).

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