THE GLYCEROL ESTER HYDROLASES OF WHEAT GERM[1]

C. E. STAUFFER[2] AND R. L. GLASS

ABSTRACT

The presence in aqueous extracts of wheat germ of three distinct esterolytic enzyme activities has been established. One, on esterase, hydrolyzes aqueous solutions of esters, and is most active toward triacetin. A second, termed a tributyrinase, hydrolyzes emulsions of esters but appears to be less active toward esters in solution. It is most active with tributyrin as a substrate. A third enzyme, a lipase, will catalyze the hydrolysis of emulsified mono-olein. Data on the enzymatic and proteinaceous characteristics of these three enzymes are presented to characterize their differences.

The increase of fat acidity during the storage of wheat is an old problem. The role of wheat lipase in the hydrolysis of lipid is not yet clear, because the nature of the enzyme itself is not well understood. That it does contribute to the increase of fat acidity was shown by the studies of Glass *et al.* (1) and of Cuendet *et al.* (2). Barton-Wright (3) also observed increases in fat acidity during storage of flour under conditions which precluded the action of noncereal (e.g., fungal) lipases. Hutchinson has written a general review (4) of the literature concerning lipolysis in stored grain.

The literature concerning wheat esterolytic enzymes has been reviewed by Geddes (5), Sullivan (6), and Reed and Thorn (7); Longenecker (8) has included wheat enzymes in a more general review of esterases and lipases. The earliest report in the literature of investigations on wheat esterase and/or lipase appears to be that by Sullivan and Howe (9) in 1933. The most extensive work on the esterase of wheat germ was that of Singer (10) and Singer and Hofstee (11,12). They partially purified an enzyme from wheat germ and characterized it as to pH optimum, heat and pH stability, and substrate specificity. Dirks *et al.* (13) made a comparative study of this enzyme and two esterases of fungal origin with respect to heat stability, sensitivity to various inhibitors, and effect of ionic strength on activity. Mounter and Mounter (14) studied the substrate specificity of a commercial wheat germ esterase.

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[2]Present address: The Procter & Gamble Co., 6000 Center Hill Road, Cincinnati, Ohio 45224.

Reed and Thorn, in their review of enzymes in wheat (7), state that "the possibility that more than one lipolytic enzyme occurs in wheat does not appear to have been investigated." To examine such a possibility, a method of differentiating between discrete enzymes is needed. For the study of esterolytic enzymes, a reliable method of discrimination of esterase and lipase is required. The earlier definition of these enzymes was not precise in terms of substrate structure. Thus, Desnuelle in 1951 (15) defined esterases as the enzymes which best hydrolyzed esters of short-chain fatty acids with monohydroxy alcohols. Lipases were most active toward esters of long-chain fatty acids with glycerol.

A later, more exact differentiation between esterase and lipase was based on the physical state of the substrate. Desnuelle and co-workers (16,17) purified pancreatic lipase and found that it exhibited no activity toward a solution of methyl butyrate. However, when methyl butyrate was emulsified with water, it was readily hydrolyzed. Thus, a more recent classification (18) of fatty acid esterases includes "esterases acting on substrates in solution (esterase proper) and esterases acting predominantly on undissolved substrates (lipase-type esterases)."

The present work was directed toward a better understanding of the lipase of wheat germ, and a clearer delineation of the various esterolytic enzymes in wheat germ, taking full cognizance of the differentiation of these two enzymes as proposed by Desnuelle (16).

Materials and Methods

Assay of Enzymatic Activity. Two types of enzymatic assay were used: a titrimetric procedure, and one based on the colorimetric determination of glycerol liberated from a glyceride substrate. The first method used a Radiometer pH-stat (Titrimeter and Titrigraph) to maintain a constant pH in the reaction mixture. The mixture consisted of 5 ml. of substrate in a water-jacketed container maintained at the desired temperature, and 1 ml. of enzyme. The rate of base addition was plotted by the Titrigraph. This rate, corrected for the uptake of base due to nonenzymatic hydrolysis of the substrate and expressed as μeq. of base per min., was directly proportional to the amount of enzyme added.

For the standard assay of esterase by this procedure, the substrate was 0.24*M* triacetin (Fisher Scientific Co., reagent grade) in water, with the reaction carried out at 37°C., pH 7.2, maintained by the addition of base. The standard assay for tributyrinase employed as a substrate an emulsion of 10 ml. tributyrin (Fisher Scientific Co., reagent grade) in 90 ml. of neutral 10% gum acacia solution. In this case the assay was performed at 20°C., pH 6.6. One unit of enzyme in either case is de-

fined as that amount of enzyme which catalyzes the hydrolysis of 1 μeq. of ester per min. under the specified conditions.

The second method of assay employed was essentially that of Luchsinger *et al.* (19). In this procedure the amount of glycerol liberated from a monoglyceride substrate is measured by oxidation with sodium periodate and colorimetric determination of the formaldehyde formed after treatment with chromotropic acid. Mono-olein (Distillation Products Industries) was dissolved in an equal weight of olive oil, and 5 g. of this was emulsified with 0.05 g. sodium taurocholate and 95 ml. of hot distilled water by mixing in a Stein laboratory mill for 5 min. One volume of the emulsion was mixed with 2 volumes of universal buffer, pH 7.8 (20), and 1 ml. of this in a 13-mm. test tube was brought to 37°C. in a constant-temperature bath. At zero time 0.4 ml. of enzyme at 37°C. was added and the mixture was incubated for 60 min.; then 0.4 ml. of a solution of 10% trichloroacetic acid (TCA) and 5% calcium chloride was added. The contents of the tube were filtered, and 0.1 ml. of the clear filtrate was assayed for free glycerol as described by Luchsinger *et al.* (19). A blank determination was carried out by adding the enzyme to the buffered emulsion at the end of the 60-min. period just prior to addition of the TCA solution. One unit of enzyme is defined as that amount which results in an absorbance at 570 mμ of 0.1, when the measurements were made in 18-mm. cuvets in a Coleman Junior spectrophotometer, and corrected for the absorbance obtained from the blank. This unit is equivalent to the release of 6.45×10^{-3} μmoles of glycerol per min. in the incubation mixture.

Determination of Protein. Quantitative protein determinations were made using the modification by Lowry *et al.* (21) of the Folin-Ciocalteu procedure (22). A standard curve was constructed with solutions containing known amounts of bovine serum albumin, and the absorbance obtained from an unknown solution was converted to protein concentration by reference to this curve.

Enzyme Purification. In the course of purification, three different enzymes were obtained. For clarity, the operational definition and names applied to each of these enzymes will be given at this point. The justification for these differentiations appears in the section on experimental results. Esterase is defined as the enzyme which hydrolyzes triacetin in aqueous solution, using the titrimetric procedure to measure the rate of hydrolysis. Tributyrinase is the enzyme which hydrolyzes tributyrin which is emulsified in water, also using the titrimetric method. Lipase is the enzyme which liberates glycerol from monoolein by the assay method of Luchsinger *et al.* (19). The latter two enzymes both hydrolyze esters in the emulsified state and therefore belong

to that class which Hofstee called "lipase-type esterases" (18). The use of the term *lipase* for only one of the enzymes in this paper is a matter of convenience, and the two names lipase and tributyrinase reflect differences in substrate specificity which are documented below.

The enzymes were obtained by fractionation of an aqueous extract of wheat germ. One part of hexane-extracted wheat germ (gift from General Mills, Inc., Minneapolis), ground in a Wiley mill to pass a 40-mesh screen, was stirred with 10 parts of distilled water for about 20 min. at room temperature. After centrifugation in an International No. 2 centrifuge at 2,000 r.p.m. for 10 min., the residue was discarded and the rather turbid supernatant was adjusted to pH 5.5 with dilute acetic acid. Centrifugation at 2,000 r.p.m. for 30 min. removed the precipitate, which contained the lipase. The supernatant was adjusted to pH 6.8 with dilute ammonium hydroxide and brought to 0.4 saturation in ammonium sulfate by the addition of 240 g. of solid salt per liter of liquid with continuous slow stirring. The precipitate, removed by centrifugation at 2,000 r.p.m., contained the tributyrinase. To the supernatant additional solid ammonium sulfate (242.4 g. per liter of original volume) was added with stirring to give a 0.7 saturation level. The esterase was precipitated by this concentration of salt, and was collected by centrifugation under the same conditions as before. The three precipitates were treated separately to further purify the respective enzymes.

Lipase. The acid precipitate, containing the lipase, was suspended in 0.1*M* Na_2HPO_4 equal to one-tenth the volume of the original extract and dialyzed against cold distilled water for 24 hr. The dialyzed solution was centrifuged at 30,000 $\times$ *g* (Servall type SS-1 centrifuge) for 10 min. to yield a yellowish opalescent solution, used for the studies reported below. A typical preparation from 15 g. of wheat germ contained 120 units of lipase activity and 800 mg. of protein. The acid precipitate contained a large amount of carbohydrate material which contributed to extremely high blank absorbances in the assay. The dialysis step resulted in a marked reduction in blank values. Other purification procedures which were applied to this preparation were unsuccessful.

Tributyrinase. The first salt precipitate, containing the tributyrinase, was suspended in distilled water equal to one-tenth the original extract, dialyzed overnight against distilled water, and centrifuged at 30,000 $\times$ *g* for 10 min. The cloudy supernatant was assayed for enzyme and protein content and treated with calcium phosphate gel, prepared according to Dixon and Webb (23, p. 51). Gel equal to 4% of the weight of protein present was stirred into the supernatant, and

then removed by centrifugation at 2,000 r.p.m. for 5 min. The supernatant was assayed for tributyrinase activity and then treated with an additional 4% of gel. This process was repeated eight to ten times until 90% of the enzyme had been adsorbed.

The gel fractions containing the first 10% of enzyme adsorbed were discarded, and the fractions containing the next 80% of the enzyme adsorbed were combined. This gel was washed five times with an equal volume of distilled water, and the washings were discarded. The gel was suspended in an equal volume of 0.1*M* phosphate buffer, pH 7.8, and allowed to stand 5 min., and the eluate was removed by centrifugation. This was repeated for a total of five elutions with each eluate kept separate. The eluates were dialyzed against cold distilled water, assayed, and lyophilized. The results of a typical tributyrinase purification procedure are given in Table I.

TABLE I

SUMMARY OF PURIFICATION OF WHEAT GERM TRIBUTYRINASE

STEP	TOTAL UNITS [a]	SPECIFIC ACTIVITY [b]	PURIFICATION	YIELD
				%
Extraction	1,492	0.036	1.0	100 [c]
Supernatant from acidification	1,186	0.042	1.2	79
Dialyzed salt precipitate	784	0.35	9.6	52
Gel eluate				
1	279	1.36	37.8	18.7
2	162	1.07	29.8	10.8
3	73	1.00	27.8	4.9
4	26	0.57	15.8	1.7
5	9	0.40	11.1	0.6

[a] One unit is the amount of enzyme which will liberate 1 μeq. of butyric acid per min.
[b] Units of enzyme per mg. protein.
[c] The amount of tributyrinase in the aqueous extract is taken as 100%, although it may not represent all the enzyme present in the wheat germ itself.

The best yield and highest-purity enzyme was obtained in the first eluate, with decreasing yield and purity in subsequent eluates. The specific activity in these could be increased by adjusting them to pH 5.3 and centrifuging to remove the precipitate which formed. One such experiment is summarized below.

Purification of Tributyrinase by Acid Treatment

	Enzyme *units/ml.*	*Protein* *mg./ml.*	*Specific Activity*
Impure tributyrinase	0.553	2.20	0.25
After adjustment to pH 5.3, supernatant	0.458	0.78	0.59

The extent of purification by this procedure ranged from 1.5- to 3-fold

with various eluates, depending on their initial purity.

Esterase. The second salt precipitate from the wheat germ extract was dissolved in distilled water equal to one-fifth the original volume of the extract. The solution was adjusted to pH 8.0, and sufficient saturated aqueous ammonium sulfate, also at pH 8.0, was added to yield a final saturation of 0.39. (In calculating the amount of solution to add, allowance must be made for the amount of 0.7 saturated ammonium sulfate solution contained in the precipitate.) The precipitate was removed by centrifugation and discarded. The supernatant was raised to 0.55 saturation by the addition of more saturated ammonium sulfate solution. The precipitate, containing the esterase, was dissolved in a minimum amount of distilled water and dialyzed to remove salts. The purification and yield of esterase obtained are given in Table II.

TABLE II
SUMMARY OF PURIFICATION OF WHEAT GERM ESTERASE

STEP	TOTAL UNITS[a]	SPECIFIC ACTIVITY[b]	PURIFICATION	YIELD
				%
Extraction	1,971	0.048	1.0	100
Supernatant from acidification	1,410	0.050	1.04	71
Precipitation between 0.4 and 0.7 Saturated $(NH_4)_2SO_4$	1,208	0.087	1.81	61
Precipitation between 0.39 and 0.55 Saturated $(NH_4)_2SO_4$	623	0.111	2.32	32

[a] One unit is the amount of enzyme which will liberate 1 μeq. of acetic acid per min.
[b] Units of enzyme per mg. protein.

The purified material may be lyophilized for storage, but requires 0.5% aqueous NaCl for re-solution. Moving-boundary electrophoresis of this preparation indicated rather gross heterogeneity, with at least five components in evidence at pH 6.9.

Experimental Results

The enzymes obtained from the purification procedures given above were characterized with respect to their enzymatic properties. The results of these studies in every case confirmed that what we have called lipase, tributyrinase, and esterase are, in fact, distinct enzymes.

Optimum pH. The optimum pH of hydrolysis was determined in the usual fashion. For the esterase and tributyrinase assays this merely involved maintaining the pH at the desired value, using the pH-stat. For the lipase a universal buffer was used which was adjusted to the desired pH by the addition of HCl before it was mixed with the emulsion. The results are shown in Fig. 1. The rates are expressed as a

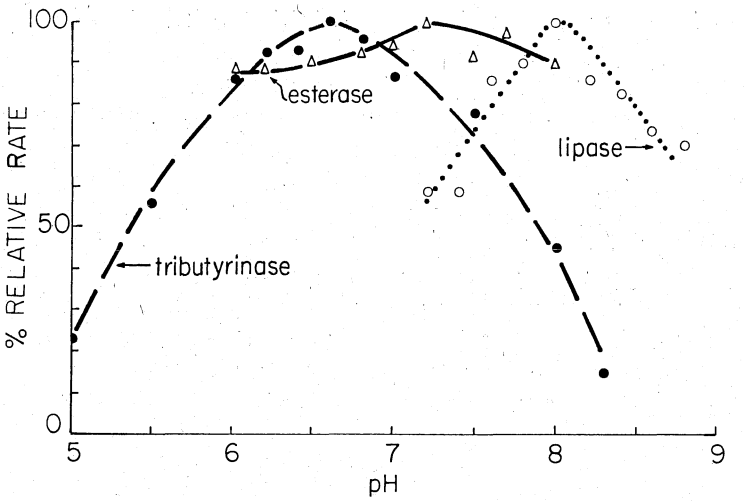

Fig. 1. Effect of pH on enzymatic activity of lipase, tributyrinase, and esterase. Lipase activity measured by the method of Luchsinger *et al.* (19), varying the pH of the universal buffer used. Tributyrinase and esterase activity toward tributyrin in emulsion and triacetin in solution respectively, varying the pH by changing the setting of the pH-stat used to follow the rate of hydrolysis. Rates are expressed as percentage of the maximum rate observed.

percentage of the maximum rate observed for each enzyme. The marked difference in the optimum pH for each of these three enzymes is readily apparent.

The esterase has a broad, flat rate *vs.* pH curve with a possible maximum at pH 7.2, in good agreement with the value of 7.2–7.3 found by Dirks (24). The flat curve is perhaps a reflection of the several components shown by electrophoresis to be present. Tributyrinase has a pH optimum of 6.6–6.8, with a fairly rapid decline in rate at either higher or lower pH values. This roughly corresponds to the pH optimum observed by Sullivan and Howe (9) when the action of aqueous wheat extracts on insoluble triglycerides was studied. Lipase has a pH optimum of 8.0, more than a full unit higher than does tributyrinase. Luchsinger (25), using a mixture of mono-, di-, and tri-olein, had determined the pH optimum to be 7.4–7.6; Koch *et al.* (26) had obtained a pH optimum of 7.9 for wheat lipolytic action on a butter-fat emulsion.

Heat of Inactivation. The denaturation of enzymes by heat is a consequence of their proteinaceous character. It can also serve as an indicator of the nonidentity of two different proteins.

Singer and Hofstee (12) reported that the denaturation of wheat germ esterase followed second-order kinetics during denaturation, and

this was confirmed by Dirks (24). The second-order rate constants for this process were determined as follows. An aqueous solution of esterase containing approximately 5 mg. of protein per ml. was adjusted to pH 7.2, and 2.5-ml. aliquots were dispensed into 13 × 100-mm. tubes. Two tubes were placed in a constant-temperature bath at the desired temperature (44°–54°C., ± 0.2°) at zero time. After 2 min., one tube was removed and immediately cooled in an ice bath. After 12 min., the second tube was removed and cooled. The esterase activity in both tubes was measured, and the rate constant for the denaturation, k, was determined by using the integrated form of the second-order rate equation:

$$k = \frac{1/C_2 - 1/C_1}{t_2 - t_1}$$

Placing both tubes in the bath simultaneously and removing them after different intervals removed the deviations in the rate of inactivation due to the length of time necessary to warm the solutions to the given temperature. From an Arrhenius plot of log k *vs.* 1/T an energy of activation of 53.4 Kcal./mole (at pH 7.2) for the denaturation process was calculated. Singer and Hofstee (12) obtained values of 65.1 Kcal./mole at pH 6.8 and 56.0 Kcal./mole at pH 7.5.

The heat-inactivation of tributyrinase, in contrast to that of esterase, is a first-order process. By following this process over the temperature range of 50°–60°C. at pH 6.6, the energy of activation for the denaturation of tributyrinase was found to be 61.8 Kcal./mole. This is similar to the value for esterase, the main distinction being the difference in the kinetics of the two processes, as well as the fact that tributyrinase is more stable than esterase. Thus, after 10 min. at 50°C., a sample of esterase had lost 51% of its activity, whereas a solution of tributyrinase had decreased only 8% in its activity. The kinetics of heat-denaturation of the lipase were not studied.

An interesting facet of the heat-inactivation of tributyrinase was the observation that it was much more labile in the presence of emulsions than it was in aqueous solutions. Thus at 30°C., pH 6.6, in the assay medium of an emulsion of tributyrin, the enzyme lost 33% of its activity in 10 min. as compared to a loss of 8% when heated in a solution. The inactivation was a first-order process. Analysis of the temperature-dependence of the denaturation gave an energy of activation of 19.5 Kcal./mole, as compared to 61.8 Kcal./mole for denaturation in an aqueous solution.

That this effect is due to denaturation of the enzyme and is not the result of a change in the state of the substrate or of product inhibi-

tion was shown experimentally. Tributyrinase solution (1 ml.) was mixed with 5 ml. of tributyrin emulsion at pH 6.6, 37°C., and the rate of butyric acid liberation was followed with the pH-stat. After 20 min., 1 ml. additional of enzyme was added to the reaction mixture, and the rate of tributyrin hydrolysis was followed for 20 min. additional. The rates of hydrolysis, as determined from the tangent to the plot of base uptake *vs.* time, are given below.

Loss of Tributyrinase Activity in an Emulsion at 37°C., pH 6.6

Enzyme Addition	*Rate*		
	Initial	*10 min.*	*20 min.*
	μeq./ml.	*μeq./ml.*	*μeq./ml.*
First ml.	0.522	0.243	0.073
Second ml.	0.463	0.236	0.135

From these results it was concluded that the tributyrin micelles are not changed in such a fashion as to render them resistant to enzymatic hydrolysis. Also, the initial activity shown by the second ml. of enzyme would discount product inhibition as the reason for the decrease in rate with time. The temperature-dependence of this inactivation was such that at 20°C. the enzyme did not decrease appreciably in activity for at least 10 min.

Substrate Specificity—Physical State of Substrate. Desnuelle and coworkers (16,17) have used the physical state of the substrate as a criterion for differentiating esterolytic activity. The choice of a water-soluble substrate, triacetin, and water-insoluble substrate, tributyrin, was a result of this differentiation.

The requirement of tribuytrinase for an emulsified substrate is shown in Fig. 2. The ethyl butyrate used was water-soluble to the extent of 0.56%. When tributyrinase was added to a solution of ethyl butyrate, no detectable hydrolysis occurred. However, when somewhat more ethyl butyrate was added and emulsified in the saturated solution, the addition of tributyrinase resulted in a measurable release of butyric acid. The rate of hydrolysis *vs.* substrate concentration curve is very much like the curve normally observed with enzymes, and one can even obtain a value for "K_M" of 1.4%. It may be thought that the only reason for the lack of observable activity in the soluble region (0.56% substrate) is that the low level of substrate will not permit a measurable rate of hydrolysis. However, using a K_M of 1.4% and a V_{max} of 0.26 μeq. per min. (obtained from an Eadie plot of the data), one may calculate from the Michaelis-Menten equation, $v = V_{max} \cdot S/(K_M + S)$, that at 0.5% substrate the rate should be 0.068 μeq. per min. This is well above the lower limits of measurable rates.

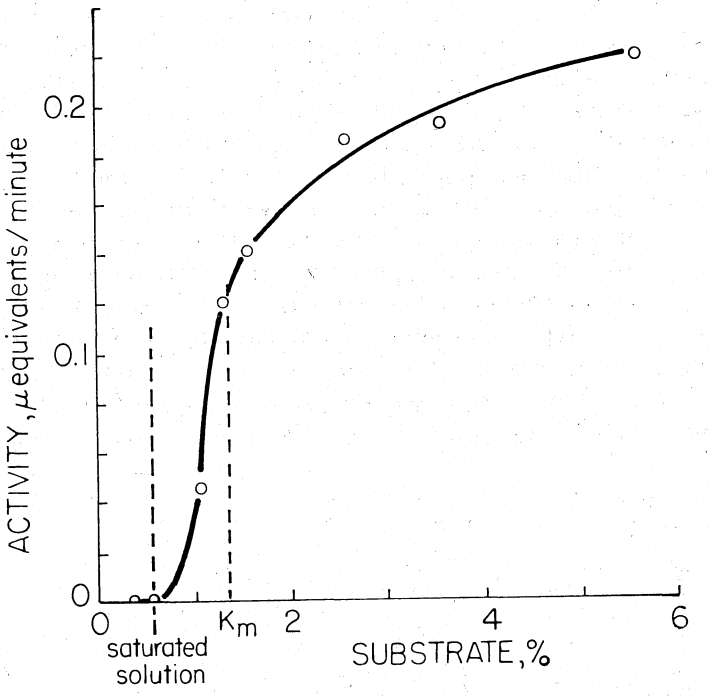

Fig. 2. Activity of tributyrinase in absence and presence of an oil-water interface. Measured at pH 6.6, 25°C., with ethyl butyrate as the substrate. Solubility of ethyl butyrate in water is 0.56% at this temperature.

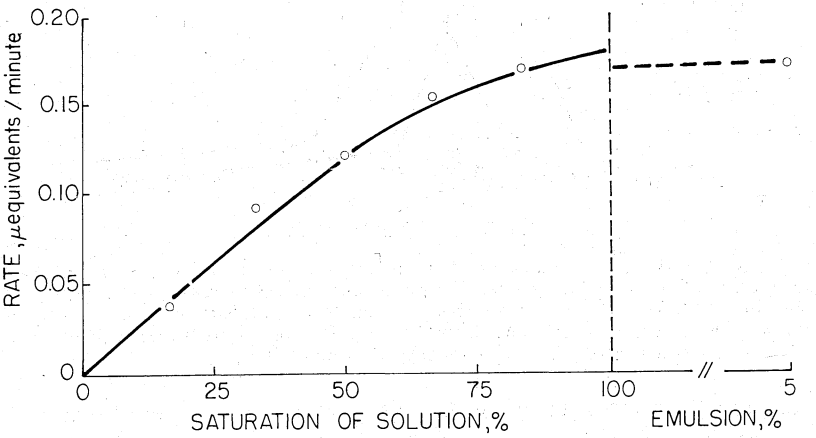

Fig. 3. Activity of esterase in absence and presence of an oil-water interface. Measured at pH 7.2, 37°C. Solutions of substrate to left of vertical dashed line prepared by dilution of a saturated solution. The emulsified substrate for the point to the right of the line was prepared by emulsifying 5 g. of ethyl propionate in 100 ml. of saturated solution.

The esterase, in contrast, showed the usual response to concentration of substrate in solution (Fig. 3). In the instance shown, with ethyl propionate as the substrate, the inclusion of substrate in excess of that amount necessary to saturate the solution (i.e., forming an emulsion) did not result in any increase in rate of hydrolysis, which is just opposite to the behavior displayed by tributyrinase. It is not felt that these data preclude the possibility that esterase hydrolyzes emulsified substrates. Other data, in fact, indicate that it does. However, the fact that tributyrinase and lipase are apparently quite inactive against dissolved substrates while esterase is very active against, for example, solutions of triacetin, is a rather strong point of differentiation between the enzymes.

Substrate Specificity — Chemical Nature of Substrate. The rate of hydrolysis of homologous series of triglycerides and ethyl esters by tributyrinase is presented in Fig. 4. The rates are given as percentages

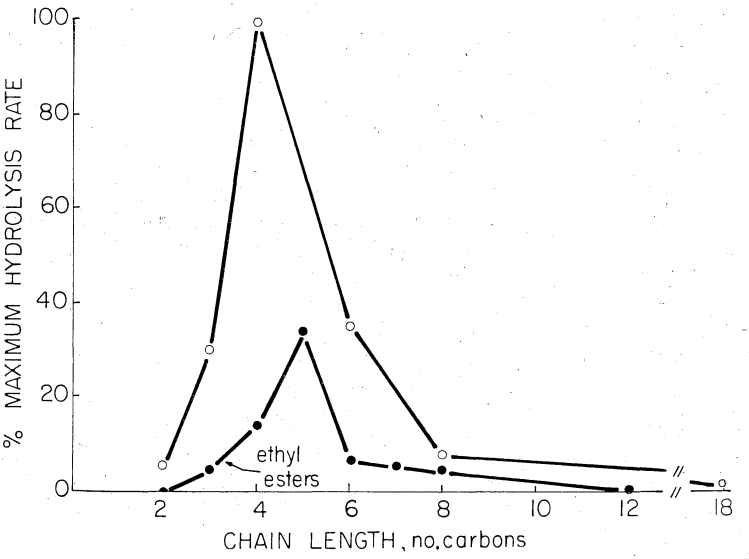

Fig. 4. Influence of fatty-acid chain length on rate of hydrolysis of triglycerides and ethyl esters by tributyrinase. Activity measurements performed at pH 6.6, 20°C., by the titrimetric method. Substrates were made by emulsifying 10 g. of ester with 90 ml. of neutral 10% gum acacia solution. The rates of hydrolysis are referred to that of tributyrin which has been assigned a value of 100.

of the maximum rate, obtained with tributyrin as the substrate. The substrates were prepared by emulsfying 10 g. of substrate with 90 ml. of neutral 10% gum acacia in water. The optimum fatty-acid chain length was four carbons for the triglycerides and five carbons for the ethyl esters. The triglycerides were hydrolyzed at about five times the

rate of corresponding ethyl ester. The extremely low rate of hydrolysis of triolein emphasizes the marked specificity of tributyrinase for esters of short-chain fatty acids.

The lipase, which was examined only in its crude form, did not show appreciable hydrolysis of short-chain triglycerides. The difference between the specificity of the two lipases is demonstrated by the data below, for which 1 mg. tributyrinase or 60 mg. crude lipase was assayed

Enzyme	*Butyric Acid Released from Tributyrin*	*Glycerol Released from Mono-olein*
	μmoles/min.	*μmoles/min.*
Tributyrinase	0.458	0.002
Crude lipase	0.000	0.058

as described under "methods." This clearly shows that the crude lipase has a relative specificity for long-chain esters.

The substrate specificity for esterase has been examined and reported by Singer and Hofstee (11,12) and Mounter and Mounter (14). It was not extensively examined in the present study, but some determinations of the Michaelis-Menten dissociation constant and V_{max} were made for purposes of comparison with the specificity of tributyrinase. The results are presented in Table III. From the trends in the sub-

TABLE III

SPECIFICITY OF WHEAT GERM ESTERASE AND TRIBUTYRINASE

SUBSTRATE	ESTERASE	V_{max} [a]	TRIBUTYRINASE RELATIVE RATES [b]
	K_m, *mM*		
Triacetin	15.2	102	5
Tripropionin	1.5	25	30
Tributyrin	...	...	100
Monoacetin	4.9	43	
Ethyl acetate	148	32	0
Ethyl propionate	52	6.9	4

[a] Moles $\times 10^9$ hydrolyzed/min./mg. protein.
[b] Rate of activity toward 10% emulsions, taking activity toward tributyrin as 100% (Fig. 4).

strate specificities of the two enzymes, it is apparent that they are different. Esterase hydrolyzes triacetin four times as fast as it does tripropionin; tributyrinase hydrolyzes tripropionin six times faster than triacetin. Ethyl acetate is hydrolyzed by esterase at four times the rate of the propionate ester, whereas tributyrinase does not hydrolyze ethyl acetate at a measurable rate, but hydrolyzes ethyl propionate at about the same rate as it does triacetin.

Discussion

The presence of several distinct esteratic enzymes in aqueous extracts of wheat germ is clearly established by the experimental results given above. Some of these different properties may be briefly summarized as follows.

The three enzymes may be separated rather sharply by the procedures described. Though this in itself is not a criterion of nonidentity, if one follows the precipitation of triacetin-hydrolyzing activity and of tributyrin-hydrolyzing activity as a function of ammonium sulfate concentration, these two activities are removed from solution at different salt concentrations, and behave like different proteins.

Esterase has a pH optimum of 7.2, with very little decrease in rate at pH values between 6.0 and 8.0. Lipase has a pH optimum of 8, whereas tributyrinase has an optimum at 6.6–6.8. Both of these enzymes decrease markedly in activity as the pH is raised or lowered from the optimum.

The heat-inactivation studies also emphasize the differences between the enzymes. The rate of esterase denaturation follows second-order kinetics. Tributyrinase, by contrast, follows first-order kinetics during its inactivation by heat. Also, tributyrinase and lipase have different stabilities to heat in the presence of oil-water interfaces. While tributyrinase is rapidly inactivated at temperatures as low as 25°C., lipase is quite stable for at least 1 hr. at 37°C., since these are the conditions for its assay, and the time-course of glycerol liberation has been shown to be linear (19,25).

Finally, the substrate specificities, both as to physical state and fatty-acid chain lengths, serve to differentiate the enzymes. Tributyrinase requires the presence of an interface for its catalytic activity, while esterase will hydrolyze esters in solution. The three enzymes have different specificity requirements with respect to fatty-acid chain length: esterase hydrolyzes acetate esters most rapidly, tributyrinase is most effective on four- or five-carbon fatty-acid esters, and lipase is most active toward mono-olein.

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