CASTOR BEAN LIPASE ACTIVATORS

by

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A Thesis Submitted to the Faculty of the

COMMITTEE ON AGRICULTURAL BIOCHEMISTRY AND NUTRITION

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

1970

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Date

ACKNOWLEDGMENTS

The author wishes to express sincere appreciation:

To Dr. Henry W. Kircher for his many helpful suggestions during the course of this work and preparation of this thesis.

To Drs. Arthur R. Kemmerer and Mitchell G. Vavich for their advice and for reading the manuscript.

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ABSTRACT

Castor bean lipase is activated by two different fractions obtained from the castor bean. It is difficult to distinguish whether these fractions are true activators or are effective in altering physical properties of the substrate. Lipase activity was found to be sensitive to high pH and rates of lipolysis were dependent on the rate of agitation of reaction mixtures. Lipase which had been exposed to high pH exhibited decreased rates of lipolysis that could be enhanced by increased agitation or by addition of a Tris soluble fraction isolated from the bean. Lipase which was not exposed to alkaline pH had higher rates of lipolysis which were independent of agitation rates.

The lipase is activated by sulfate as well as molybdate ion.

A fluorescent assay was developed which suggested that there may be more than one hydrolytic enzyme in the preparation.

INTRODUCTION

Crude lipase activity was first observed in castor bean seeds (Ricinus communus) by J. R. Green in 1890 (1). He was investigating the nature of biochemical compounds in oil-containing seeds that were responsible for the seed's energy source during germination. Green postulated that the oil was transformed into starch and utilized along pathways similar to those in starch storing seeds. He was able to isolate a "ferment" from the castor seed that hydrolyzed the endogenous triglycerides to their component fatty acids. This, he maintained, was the first event in the breakdown of the stored fats to the energy required for germination.

In the investigations that followed, notably those of Longenecker and Haley (2), the general properties of the lipase were elucidated. He found it to be an acid lipase with maximal activity occurring at pH 4.2 and relatively nonspecific toward the fatty acid component or its position in a triglyceride. The lipase is a particulate enzyme and is insoluble at its physiological pH. Attempts to solubilize the lipase have been unsuccessful (3). The predominant fatty acid present in the natural substrate is ricinoleic acid. However, Altschul, Ory and St. Angelo have determined that maximal activities are observed with

triglycerides composed of short, saturated ester side chains of four to eight carbons. Hydrolysis of unsaturated triglycerides containing 16 to 18 carbon ester side chains is faster than saturated triglycerides containing 12 carbon ester side chains. But both these rates are slower than those observed with the short chained, saturated triglycerides. In addition, these preparations exhibit decreasing rates of hydrolysis with tributyrin>dibutyrin>monobutyrin; but in contrast, the rate of hydrolysis of diolein is faster than that of triolein. These results led to speculation that there were two lipolytic species present in his crude preparations. The first cleaved short chained, saturated esters and the second species hydrolyzed long-chained, mixed or saturated esters from the triglyceride (4). Savary, Flanzy and Desnuelle found that there was no positional specificity by the lipase and that each position of the triglyceride was acted upon with equal facility (5). Longenecker and Haley indicated that the lipolytic reaction occurs as an oil-in-water emulsion and no emulsifying agent was required for maximal activities (2). This is in contrast to other lipase systems such as the phospholipases that have been shown to utilize cardiolipin, inositolphosphatides and certain other emulsifiers as "co-factors" (6). The allergenic properties of the lipase preparation precluded any significant advances in the field until Ory was able to isolate a fat-free enzyme that was also free of the allergenic proteins. The al-

lergens have been studies extensively by Spies and Coulson (7) and also by Layton, Mors and De Eds (8). Castor bean allergens appear to be comprised of a complex mixture of proteins and glycoproteins for which no specific biochemical significance can be attributed. Kircher[1] has indicated in unpublished work that the allergens may be linked with activation of the lipase. They have shown that certain of Spies' fractions have an activating effect when added to the castor bean lipase system.

Recent investigations by Ory, Yatsu and Kircher suggest that the particulate enzyme is associated with a lipid containing body within the castor bean endosperm, the spherosome (9). Their experiments indicate the lipase is attached to the membrane surrounding the lipid body. If the isolated spherosomes are floated in a dilute lead solution under lipolytic conditions, the bodies sink as the liberated fatty acids combine to form lead soaps. Electron microscopy shows the lead soaps appear to be located on the outer edge of the spherosomes. If the enzyme is indeed attached to the membrane, this could account for the insolubility of the lipase.

Ory, Barker and Boudreaux also report the existence of a lipid co-factor associated with lipase (10). Extraction

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of the crude lipase preparation with n-butanol yielded an apoenzyme with reduced activity that could be restored upon addition of the butanol soluble fraction. They have further indicated that the identity of the co-factor to be the cyclic tetrameric ester of ricinoleic acid. Using the apoenzyme in assays comparing the rates of hydrolysis of tributyrin and long-chained, unsaturated triglycerides in the presence of the co-factor, tributyrin rates were significantly slower; again suggesting that there may be two lipolytic enzymes in the preparation.

In an attempt to purify the crude lipase preparation by incubation in buffer at pH 5.2, Ory, Kircher and Altschul detected reduced activity after centrifugation that could be restored by the addition of the soluble fraction (11). the soluble fraction had considerable different properties than that of the lipid co-factor. The activator fraction was heat stable, nondialyzable, water soluble and had no apparent enzymic activity. It moved as one band on sedimentation after passage over a DEAE-cellulose column, but showed several bands on polyacrylamide gel electrophoresis.

These investigations were initiated with this fraction with the intention of comparing the properties of the pH 5.2 fraction with the water soluble (allergenic) fraction to determine if there were common properties and to identify these activating components.

This approach led to the observation that neither the allergenic nor the CBL* supernatant fractions contained a true activator but that possibly each was participating in altering the physical properties of the substrate sufficiently to change the apparent rates of reaction of the lipase.

EXPERIMENTAL

Materials

Beans. Baker variety 296 castor beans were obtained through the courtesy of D. S. Bolley of the Baker Castor Oil Company. The beans were stored at 15°C until used.

Buffer Solutions.

TCE 7.9 A solution of 0.01 M tris(hydroxymethyl)-aminomethane (Tris), 0.05 M cysteine-HCl and 0.01 M ethylenediaminetetraacetic acid(disodium salt), (EDTA) was adjusted to pH 7.9 with 0.1 M NaOH.

TCE 5.2 Same as TCE 7.9 except the pH was adjusted to 5.2.

ACE 4.4 - 1 M acetate buffer, pH 4.4, 0.05 M cysteine-HCl, and 0.01 M EDTA.

Cottonseed Oil The cottonseed oil used as substrate was commercial Wesson Oil.

4-methylumbelliferone caproylate - (4MUC) - The fluorogenic substrate was the gift of Dr. H. W. Kircher as was the 4-methylumbelliferone (4MU).

Methods

Lipase Preparation. Enzyme isolation and partial purification was accomplished by a modification of the procedure of Altschul, Ory, and St. Angelo (12). Five hundred grams of whole castor beans were homogenized in 800 ml of TCE 7.9 buffer for 5 minutes in a large Waring blendor. The resulting thick suspension was tranferred to 250 ml centrifuge bottles and spun at 8000 rpm (10,400 g) for 30 minutes in a Sorvall GSA head. This yielded a fatty layer which floated midway between the precipitate and the meniscus of the buffer. The buffer was decanted and saved for the preparation of the Tris soluble fraction (TS). The fatty layer was spooned into a 2 liter separatory funnel. Further purification was accomplished by removal of salt soluble proteins and lipids through a partition between NaCl saturated TCE 7.9 (SNTCE 7.9) and ethyl ether. The fatty layer was shaken until a smooth suspension was observed. This mixture was centrifuged at 8000 rpm for 30 minutes. The floating particulate layer containing the lipase was isolated by decantation of the buffer-ether, transferred to the separatory funnel, washed and shaken twice more with an equal volume of SNTCE 7.9 and 100 ml ether and centrifuged each time at 8000 rpm for 30 minutes. The particulate fraction was then freed of the salt and

ether by dialysis against distilled water in the cold (15°C) for 24 hours with frequent changes of water. The non-dialyzable fraction was centrifuged at 17,000 rpm (34,800 g) for 30 minutes in a Sorvall SS34 head. The pellet was taken up in a minimum volume of distilled water and lyophilized. Five hundred grams of beans yielded 1.5 to 2 grams of castor bean lipase (CBL) containing 30-34% protein by micro-Kjeldahl analysis. See Figure 1 for a flow diagram of the procedure.

Preparation of pH 5.2 Treated CBL (CBL*). CBL was made up at a concentration of 20 mg/ml in TCE 7.9, homogenized in a Potter-Elvehjem apparatus and transferred to a 30 ml beaker. The pH was adjusted carefully to 5.2 with 0.3 M acetic acid. The mixture was capped and allowed to stand in ice for 20 minutes. The pH was quickly adjusted to 6.0 with 0.1 M NaOH and centrifuged in an International table top centrifuge for 10 minutes. The precipitate was taken up in sufficient TCE 7.9 or appropriate buffer to approximate 20 mg/ml (assuming no protein loss) and homogenized. This suspension was named CBL* and the supernatant was named Fraction 3. See Figure 1.

Preparation of the Tris Soluble Fraction. The Tris soluble fraction from the preparation of CBL was autoclaved at 125° and 15 psi for 10 minutes, allowed to cool to near

room temperature and centrifuged at 8000 rpm for 20 minutes in a Sorvall GSA head. The precipitate was discarded and the supernatant filtered through a glass wool plug and dialyzed at room temperature against distilled water for 48 hours with frequent changes of water. The non-dialyzable fraction was lyophilized to a yellowish powder. The yield was about 6 grams per 500 grams of beans and contained 88% protein by Biuret determination. See Figure 1.

Assay Techniques. The assay method utilized was essentially that of Ory, St. Angelo and Altschul (13); the back titration of the liberated fatty acids with 0.100 N NaOH.

The stock castor bean lipase suspension was usually made up at a concentration of 25 mg/ml of specified buffer. The suspension was homogenized until smooth. Prior to taking an aliquot for assay, the stock suspension was mixed for 5 seconds on a Vortex shaker to insure homogeneity of the aliquot.

In a typical assay, 0.2 ml of the CBL suspension (0.3 mg protein) was added to 1.4 ml water and 0.33 ml cottonseed oil (1000 µmol total ester bond). This mixture was homogenized in a Potter-Elvehjem apparatus for 15 seconds or until a smooth, creamy suspension was evident. The suspension was transferred to a 30 ml beaker containing a stirring bar and the homogenizer was rinsed with two 1.5 ml

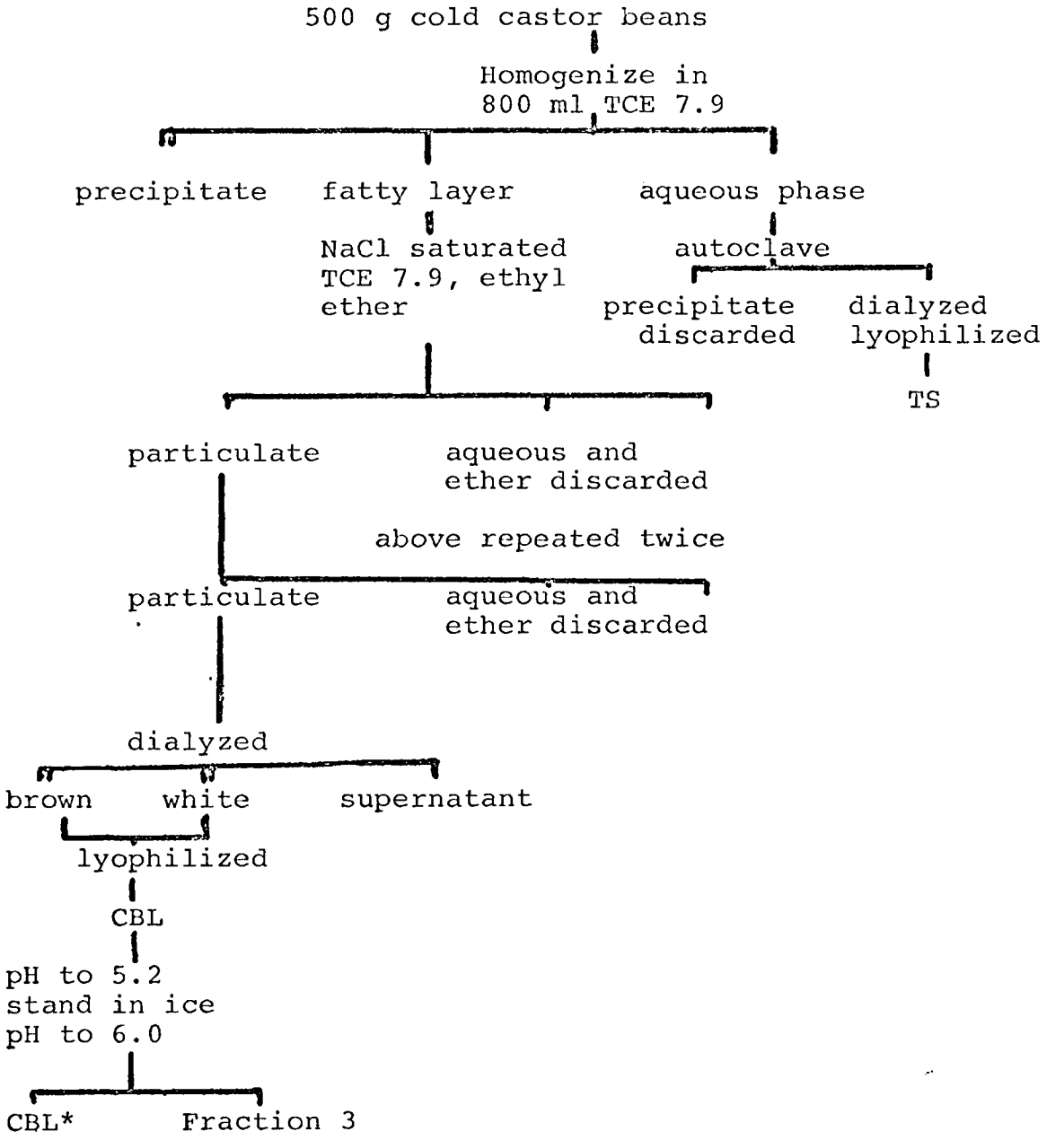

Figure 1. Preparation of CBL, CBL*, and the TS Fraction.

washes of distilled water. Final reaction volume was 5.0 ml. Stirring was started on a magnetic stirrer and continued at a sufficient rate to create a slight vortex. At zero time, a pre-determined amount of acetic acid (glacial acetic diluted 1:50, v/v) was added to lower the pH to the optimum, 4.2-4.4. The reaction was usually allowed to proceed for 10.0 minutes and was quenched with 10.0 ml absolute ethanol. The alcohol-water solution was titrated with 0.100 N NaOH to an endpoint of pH 9.25 using a Radiometer pH-stat. The titration was considered complete if the endpoint was maintained for 15 seconds. A sample containing all the additions, but stopped immediately after the acetic acid addition, comprised the blank; in other cases, all additions except enzyme were carried through the entire reaction procedure to eliminate non-enzymatic reactions from escaping detection. Rates of reaction were expressed as µmol fatty acids released mg protein minute.

A second assay method was performed by shaking the same reaction mixture in a 13 ml centrifuge tube on a Lab-Line vortex shaker with the tube clamped into position above the shaker. Five mg of the CBL suspension, 1000 µmol cottonseed oil and enough water to make 5.0 ml total volume was added to the tube. This mixture was shaken for 10 seconds to insure a homogenous suspension and a

pre-determined amount of acetic acid added to lower the pH to 4.2. The mixture was shaken for 4.0 minutes, usually at maximum speed, allowing the liquid to rise up to the top of the capped tube. The vortex shaker was stopped and 5.0 ml of absolute ethanol was added to stop the reaction. The solution was transferred to a 30 ml beaker and an additional 5.0 ml of alcohol was used to rinse the tube into the beaker for titration. Rates were expressed as above.

It became apparent in early studies of the back titration technique that reproducibility was unsatisfactory. This was probably due to such factors as rate of stirring, size of the CBL suspension aliquot, and the titration itself. Therefore, the third assay method used to determine the activities of CBL was that of following the hydrolysis of a fluorogenic compound. It was hoped that this assay would gain added sensitivity as well as reproducibility.

Jacks and Kircher reported on several fluorogenic substrates that exhibit intense fluorescence upon hydrolysis (14). One of these was the caproyl ester of 4-methylumbelliferone (7-hydroxy-4-methylcoumarin), abbreviated here as 4MUC. The ester itself has no fluorescence but upon cleavage, low concentrations of the free alcohol moeity have an intense fluorescence at

450 mμ. Fluoresence was linear with concentration over a limited range.

The reaction was followed with a Coleman Model 12A fluorometer modified in the following manner: the primary filter, B-1, which passes a wide band centered around 365 mμ, was fitted with a metal disc that had a small hole (about 1 mm in diameter) drilled into its center to allow a minimal amount of light to pass. The secondary filter, PC-1, was also fitted with a similar disc. PC-1 allows a wide band pass centered on 445 mμ. This modification was required because the intensity of emission of the fluorogenic compound was quite high at substrate concentrations used in the assay. A solution of 0.3 mg quinine sulfate per liter of 0.1 N H_2SO_4 was used to standardize the instrument.

A standard solution of 1×10^{-4} M 4-methylumbelliferone (4MU) was made up in 0.16 M acetate buffer, pH 4.4. The substrate, 4MUC, was relatively water insoluble and had to be initially dissolved in 1,2-dimethoxyethane and diluted to the desired concentration of 1×10^{-3} M with water. This was later changed to a 2-methoxyethanol-water solution because of complications presented by the dimethoxethane.

The fluorogenic assay conditions included 1.06 mmol acetate buffer, pH 4.4; 0.6 μmol 4MUC, and 0.75 mg CBL in

TCE 7.9 buffer. The total reaction volume was 6 ml. The reaction was started by addition of enzyme to all components and was followed for varying amounts of time generally dependent on the rate of reaction and the time necessary reach 100% on the instrument's scale. In some instances, the reaction mixture was stirred but in a majority of cases the rates were linear without stirring. An attempt was made to quantitatively represent the concentration of the hydrolyzed moiety by construction of standard curves; however, difficulties were encountered with drifting of the instrument, probably due to its battery-operated internal circuitry. Consequently, only estimates of the absolute concentrations of the hydrolyzed ester were achieved.

Electrophoresis. Electrophoretic studies were conducted with a Spinco Duomatic apparatus using cellulose acetate membranes as support. Barbituric acid buffer at pH 8.6 and ionic strength 0.075 was used. Solutions to be run were made up at 25% w/v in the buffer. The sample was allowed to run for 30 minutes at an initial voltage of 250v and 5 milliamps. The membranes were developed in a 1% amino black solution.

Lyophilization. Lyophilization was conducted with an Atmo-Vac table freeze dryer equipped with a

refrigeration unit. Samples were normally frozen and lyophilized within 24 hours.

Gel Filtration. Gel filtration was conducted on two columns containing Bio-Gel P-6 with an exclusion value of 4600. The first column was used for gross separation and had the dimensions 3.5 by 37 cm. Three major bands from the TS fractions were eluted with distilled water and each of these were run on the second column of dimensions 1 x 50 cm for greater resolution. Ten ml aliquots were collected from off the first column and three ml aliquots from the smaller column and recorded at 280 mμ on a Gilson Medical Electronics automatic fraction collector equipped with a Texas Instruments recorder. Each of the tubes were lyophilized and made up to desired concentrations for assay with distilled water. The amount of TS placed on the large column ranged from 50 to 200 mg and 25 mg at each peak was placed on the smaller column.

RESULTS

Enzyme. In all the preparations of castor bean lipase, the last centrifugation before dialysis yielded a bi-layered pellet. The upper layer was white in color and the lower was a brownish-gray. The specific activity of each layer was nearly identical and assayed at 4.6 and 4.3 µmol fatty acids released/ min/ mg protein for the white and brown layers respectively. Therefore, in all subsequent preparations of the lipase, both layers were combined, lyophilized and used as a homogeneous suspension in the specific buffer used in the analysis.

Published values of rates of reaction of CBL utilizing 1.5 mg enzyme protein and 1000 µmol substrate in 5 ml buffer and stirring the reaction mixture for 10 minutes have been reported to be 4.1 to 5.0 µmol ester hydrolyzed min/ mg protein (10). Rates observed here for these conditions ranged from 3.8 to 6.6 µmol fatty acids released min/ mg protein depending on the particular CBL preparation used in the analysis. See Table 1 for some typical results.

Activating Species. When CBL was treated at pH 5.2 for 30 minutes, the treated lipase (CBL*) had diminished

Table 1. Activities of CBL Preparations.

Each assay contained 1.5 mg protein, 1000 µmol cottonseed oil and sufficient acetic acid to drop the pH to 4.2 in a total volume of 5 ml which was stirred for 10 minutes. The CBL stock suspension was made up in TCE 7.9 buffer.

ENZYME PREPARATION	ADDITIONS TO ASSAY	SPECIFIC ACTIVITY	
CBL 7B (lower layer)	None	4.7	
		4.7	
		4.3	
CBL 7W (upper layer)	None	4.5	
		4.2	
		4.2	
CBL 6	None	6.6	
		5.3	
CBL 8	None	6.6	
CBL 9	None	3.8	
CBL 10	None	5.8	
CBL 15	None	6.4	
CBL 3B	None	5.3	(100%)
CBL 3B*	None	0.8	(15%)
CBL 3B*	0.3 ml Fraction 3	3.8	(73%)
CBL 3B*	0.3 ml 0.01 M TCE 5.2	2.1	(40%)
None	0.3 ml Fraction 3	0.0	(0%)
CBL 9	None	3.8	
CBL 9	0.3 ml Fraction 3	11.9	
CBL 6	None	7.7	
CBL 6	CBL washed twice with TCE 7.9	7.8	
CBL 6	1×10^{-3}M PCMB	0.0	

activity that could be restored by the addition of the supernatant (Fraction 3). The CBL* exhibited 15% of the activity of the CBL and regained 70-75% of the original activity upon addition of Fraction 3. But 40% of the original activity was also regained by the addition of an equal amount of 0.01 M TCE 5.2 replacing Fraction 3. See Table 1.

It was also found that the rate of reaction increased almost three fold when CBL rather than CBL* was run in the presence of Fraction 3. Consequently, in succeeding experiments Fraction 3 was tested against both CBL and CBL*. Specific activities remain essentially the same through three successive washes with TCE 7.9. The lipase as prepared here was 100% inhibited by 1×10^{-3} M p-chloromercuribenzoic acid (PCMB) in conformation with the literature (15). See Table 1.

Tris Soluble Fraction (TS). Activation of a CBL preparation by the Tris soluble fraction (TS) was found in agreement with an unpublished report of H. W. Kircher[2] Maximal activities were observed at 7.5 mg of the lyophilized TS. An inhibitory effect at concentrations greater than this is shown in Figure 2 where activities were plotted against concentration of TS.

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2. See footnote page three.

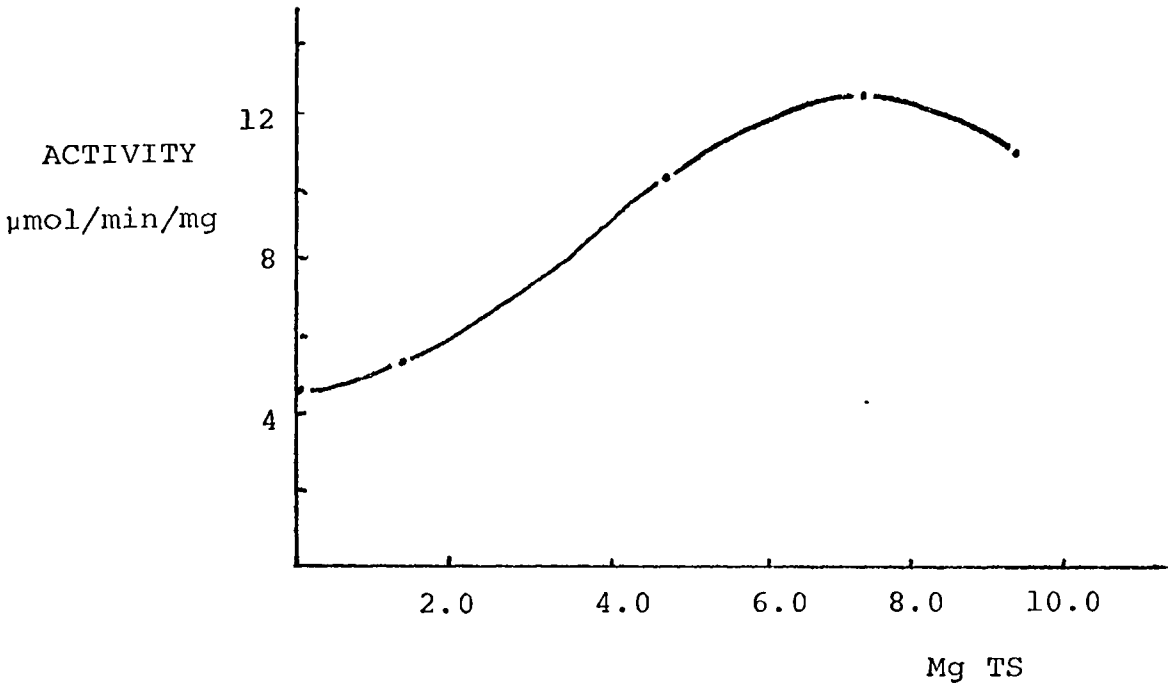

Figure 2. Activation by the TS Fraction.
Each assay contained 1.5 mg protein in TCE 7.9, 1000 µmol cottonseed oil, lyophilized TS in distilled water as noted and was stirred for 10 minutes. Total reaction volume was 5 ml.

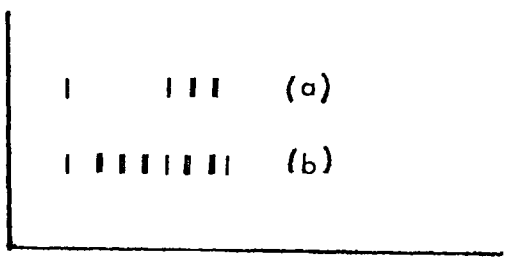

Figure 3. Electrophoresis of TSS and TS.
Cellulose acetate electrophoretic patterns run for 30 minutes at 250v and 5 milliamps. (a) TSS; (b) TS. The buffer system was barbituric acid at pH 8.6 and ionic strength 0.075. The membrane was developed in 1% amido black.

The TS fraction [(b) in Figure 3] had several bands on cellulose acetate electrophoresis and showed at least three major peaks on gel filtration through a Bio-Gel P-6 column. Fractions from the column corresponding to tube numbers 15-24, 25-27, and 28-32 in Figure 4 were collected and lyophilized. Each of these groups was run on a second, smaller column to improve resolution. The activity of each of the three peaks was assayed and it was found that no single peak had greater activity than the original TS fraction, although the earlier fractions did show some activation. See Figure 5.

In another approach to fractionation, an aqueous solution of the TS fraction at pH 4.2 was saturated with ammonium sulfate to precipitate a major portion of the proteins in that fraction. This solution was stirred for one hour at room temperature and then centrifuged. After dialysis of the supernatant against running distilled water for 48 hours, the non-dialyzable fraction was lyophilized to a yellow powder (TSS). Electrophoresis of TSS indicated a diminished number of bands [(a) in Figure 3] when compared to the TS fraction. The TSS fraction when added to a preparation of CBL had considerable activity. It also showed at least four spots on a

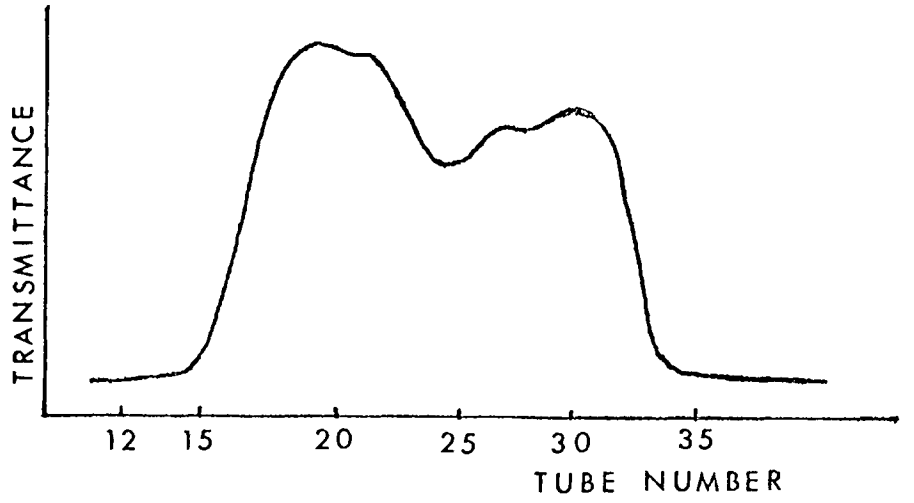

Figure 4. Gel Filtration of the TS Fraction.
The elution pattern of the TS fraction from a 3.5 x 37 cm Bio-Gel P-6 column as eluted with distilled water. Fraction volume was 10 ml.

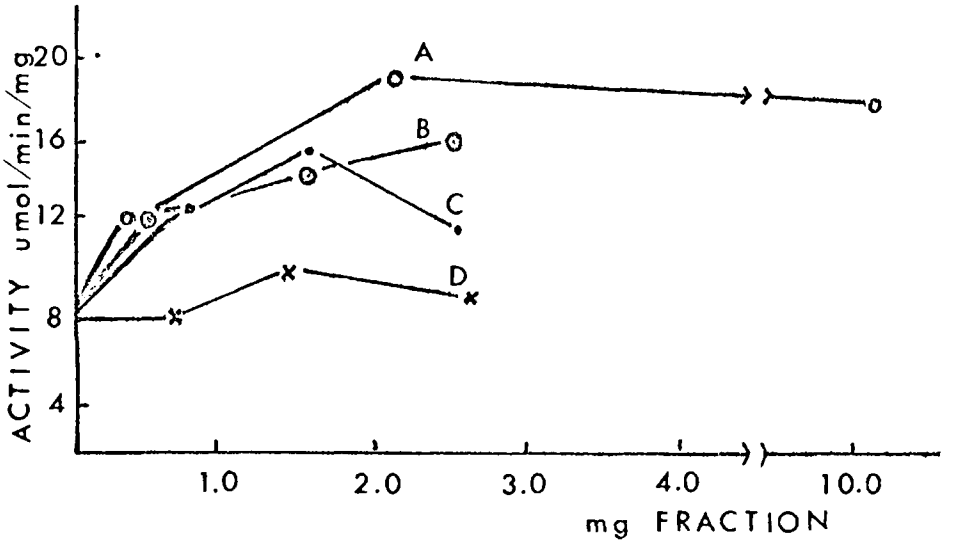

Figure 5. Activities of Fractions from Gel Column.
Each assay contained 1.5 mg protein in TCE 7.9, 1000 µmol cottonseed oil and the indicated amount of lyophilized fraction from the gel filtration column in a total reaction volume of 5 ml. A: TS fraction prior to column, B: tube numbers 25-27, C: tube numbers 15-24, D: tube numbers 28-32.

TLC plate developed in distilled water at a neutral pH. But when each spot was eluted and isolated from the silica gel, each proved to have nearly identical activities. Further experiments indicated that the $CaSO_4$ used as binder in the silica gel was responsible for the activity and that sulfate ion was the activating species. All sulfate salts that were subsequently tested activated CBL as did molybdate ion. Monovalent anions had no apparent activating potential. Table 2 contains a series of anions tested.

The TSS fraction was assayed for sulfate ion by perchloric acid digestion, treatment on a cation exchange resin and titration with $BaCl_2$. TSS was found to contain 16% sulfate (160 µg sulfate/mg TSS), apparently residual from incomplete dialysis after ammonium sulfate precipitation as the TS fraction assayed at only 1.7% sulfate (17µg sulfate/mg TS).

It was conceivable that the endogenous sulfate ion in the castor bean contributed to the activation of CBL. Five milligrams of the TS fraction would contain about 85 µg of sulfate ion which could account for some of the activity of the TS fraction. However, after treatment of the TS fraction with $BaCl_2$ to precipitate the sulfate ion and testing the activity of the sulfate

Table 2. Anion Activation.

Each assay contained 1.5 mg lipase protein, 1000 µmol cottonseed oil, the indicated additions in 5 ml and the reaction mixture was run on the vortex shaker for 4 minutes.

ADDITION TO ASSAY	AMOUNT (ug)	SPECIFIC ACTIVITY
None		23
		23
		24
		24
TSS	100	34
Silica Gel G	100	28
	300	37
Silicic acid	100	25
	300	22
$CaSO_4$	100	38
	300	43
$(NH_4)_2SO_4$	100	38
	300	43
$MgSO_4$	100	38
	300	42
$CaCl_2$	100	28
	300	30
NaCl	100	24
	300	26
$NaNO_3$	100	28
	300	31
Na_2CO_3	100	23
	300	30
Na_2MoO_4	100	39
	300	40

free preparation with proper controls, it was determined that endogenous sulfate contributed little, if any, to the activity of the TS fraction.

Rate Studies

CBL in Stock Suspension of TCE 7.9. All the above assays with the exception of those in Figure 5, were conducted with a CBL stock suspension made up in TCE 7.9 buffer at a concentration of 25 mg/ml. The method of agitation was to stir the reaction mixture in a 30 ml beaker with a small magnet. If, instead of stirring, the Vortex shaking method was used for agitation (see Assay Techniques), the rate profiles of CBL with and without the addition of the TS fraction changed considerably. The data in Table 3 show that there is less relative activation by the TS fraction using the Vortex method, but the overall rates are about four times higher than the comparable assays using the stirring technique. The maximum increase of lipolytic activity of CBL by addition of the TS fraction with the stirring method is about 290% (from 6.4 to 21.9 μmol fatty acids/ min/ mg protein, and only 12% using the Vortex shaker method. Rate of lipolysis by CBL in the Vortex method is linear with time as indicated in Figure 6.

Table 3. Comparison of Stirring and Vortex Methods.

Each assay contained 1.5 mg protein in either TCE 7.9 or ACE 4.4 as indicated, 1000 µmol cottonseed oil in 5 ml total volume. The reaction was run for 5 minutes.

TS FRACTION Added to assay (mg)	SPECIFIC ACTIVITIES CBL in TCE 7.9		CBL in ACE 4.4	
	Stir	Vortex Shaker	Stir	Vortex Shaker
None	6.4	27.6	50.0	40.0
0.5	11.8	32.2		
2.0	19.1	34.6		
5.0	19.5	30.2		
10.0	13.8	28.4		

CBL in Stock Suspension of ACE 4.4 . When the stock suspension of CBL was made up in ACE 4.4 buffer, the rates of lipolysis of CBL alone were higher in the stir method when compared to the Vortex shaker method. Both of these rates are higher than any rate derived with the CBL suspension in TCE 7.9. No data was available with the TS fraction added to this assay. See Table 3.

The activity of CBL decreases with successive washes with ACE 4.4 buffer using the Vortex method for assay. The activity after one wash decreased to 33.4 µmol fatty acids released/ min/ mg protein from the original 40.0 and decreased to 24.8 after a second wash. The lipase after these washes becomes very insoluble and tacky and had a tendency to accumulate around the pestle when undergoing

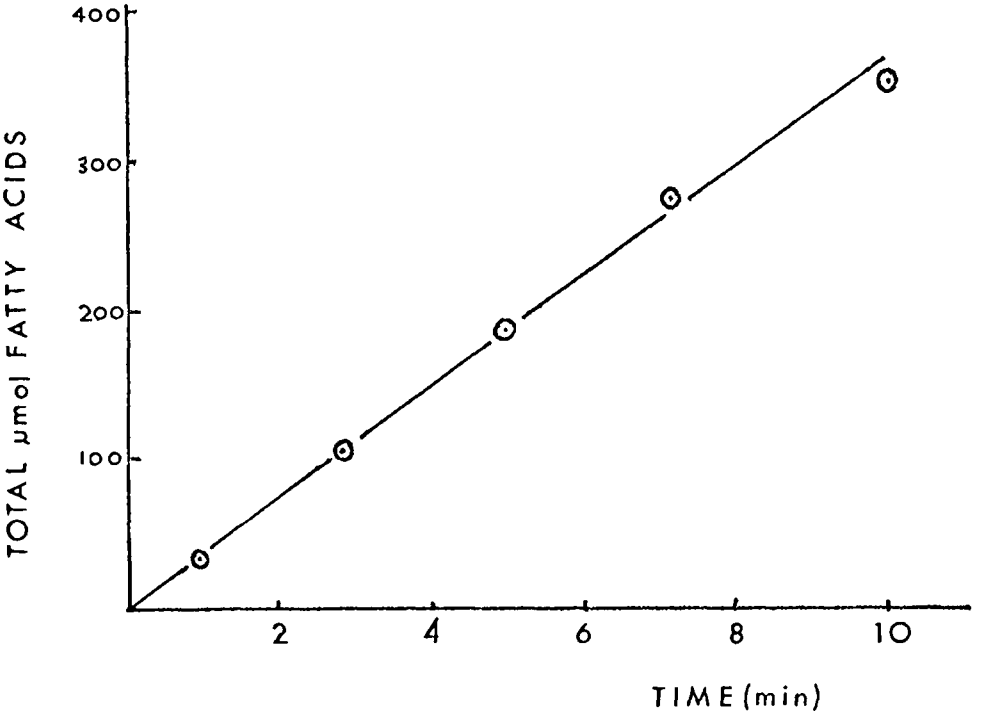

Figure 6. CBL vs Time Using Vortex Method.

Each assay contained 1.5 mg protein in TCE 7.9, 1000 µmol cottonseed oil and was agitated on a Vortex shaker for varying amounts of time as noted.

homogenization. Loss of activity may be due in part to the non-reproducibility of the lipase aliquot size or perhaps due to the hydrolysis of the lipid co-factor reported by Ory.

Modification of the CBL* Procedure. Since the activities of CBL were dependent on the pH of the stock suspension, CBL* was tested for the same effect. Normal procedure in preparation of CBL* requires adjustment of the pH 5.2 treated material to pH 6.0 after one-half hour in the cold and centrifugation. The resulting precipitate was normally taken up in TCE 7.9 for assay. However, the procedure was modified at this point by dividing the precipitate into two equal parts with one half taken up in TCE 7.9 and the second half in ACE 4.4 and the activity of each tested. The original CBL in TCE 7.9 had an activity of 4.6 µmol fatty acids released/ min/ mg protein. CBL* in TCE 7.9 had an activity of 1.4 that increased to 3.5 with the addition of 0.3 ml of Fraction 3. The activity of CBL* in ACE 4.4 was 46 µmol/ min/ mg protein. Therefore the same effect observed with CBL in ACE 4.4 was seen in CBL* in ACE 4.4; furthermore, the latter does not require Fraction 3.

Table 4. Activity of CBL* Modification.

Each assay contained approximately 1.5 mg protein in an appropriate buffer, 1000 µmol cottonseed oil and additions as noted in 5 ml total volume. The reaction was stirred for 5 minutes.

LIPASE PREPARATION	ADDITION TO ASSAY	SPECIFIC ACTIVITY
CBL 9/TCE 7.9	-	4.6
CBL* 9/TCE 7.9	-	1.4
CBL* 9/TCE 7.9	0.3 ml Fraction 3	3.5
CBL* 9/ACE 4.4	-	46.0

Fluorescence Studies. A 1×10^{-3} M solution of 4-methylumbelliferone (4MU) in 0.167 M acetate buffer, pH 4.4 was diluted to prepare standard solutions for fluorometry. The fluorescence of 4MU solutions were plotted from 0 to 600 mµmol which yielded a reasonably linear curve over this range. See Figure 7. Unfortunately, this plot could not be utilized for all subsequent assays to determine absolute rates in terms of mµmol 4MU produced by the enzymatic hydrolysis of the caproyl ester of 4MU because

of the instability and drift of the fluorometer. It was difficult to obtain reproducible settings on the instrument apparently due to the internal battery operated circuits. Consequently, some of the following rates are expressed as units of fluorescence per minute while others are in actual millimicromoles of 4MU produced per minute per mg protein.

The emission frequency of 4MU is constant in the range from pH 3 to 10, but the excitation frequency is pH dependent and varies from 330 mμ at pH 10.4 (14). In addition, as the alkalinity increases, the intensity of emitted radiation also increases for a given concentration of 4MU. This was shown by measuring the fluorescence of 20 mμmol of 4MU in 6 ml of 0.167 M acetate buffer or 0.1 M Tris buffer from pH 3.5 to 8.7. The data is plotted in Figure 8 using a wide band pass primary filter and shows this increase in intensity with increase in pH. A quinine standard was used to zero the instrument at each measurement. The high intensity of fluorescence at alkaline pH values required a second quinine standard using the same primary and secondary filter arrangement.

Linear progress curves of 4MU liberated with time at three lipase concentrations are shown in Figure 9(a) and this data is replotted in Figure 9(b) as lipase

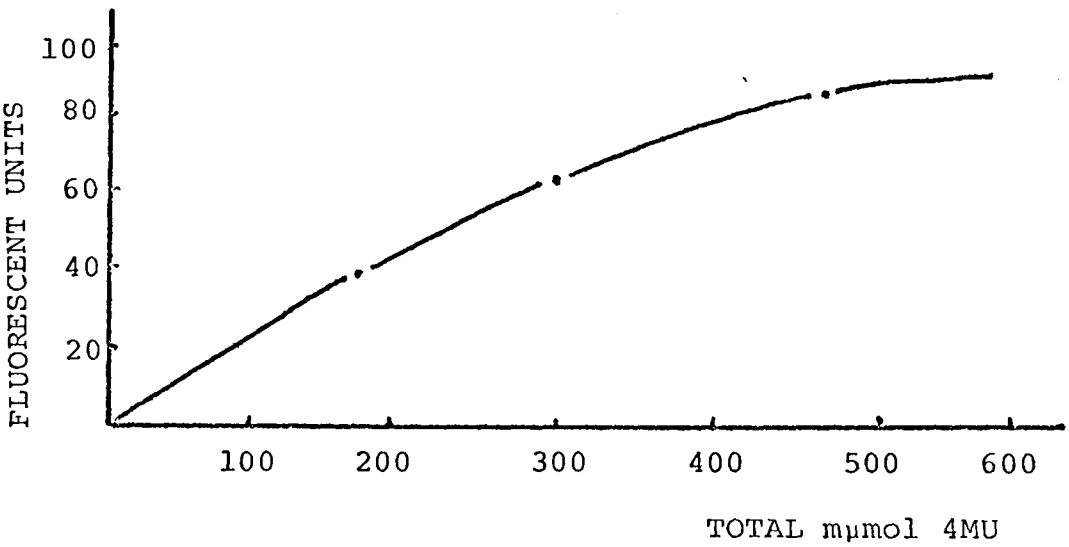

Figure 7. Standard 4-Methylumbelliferone Curve.

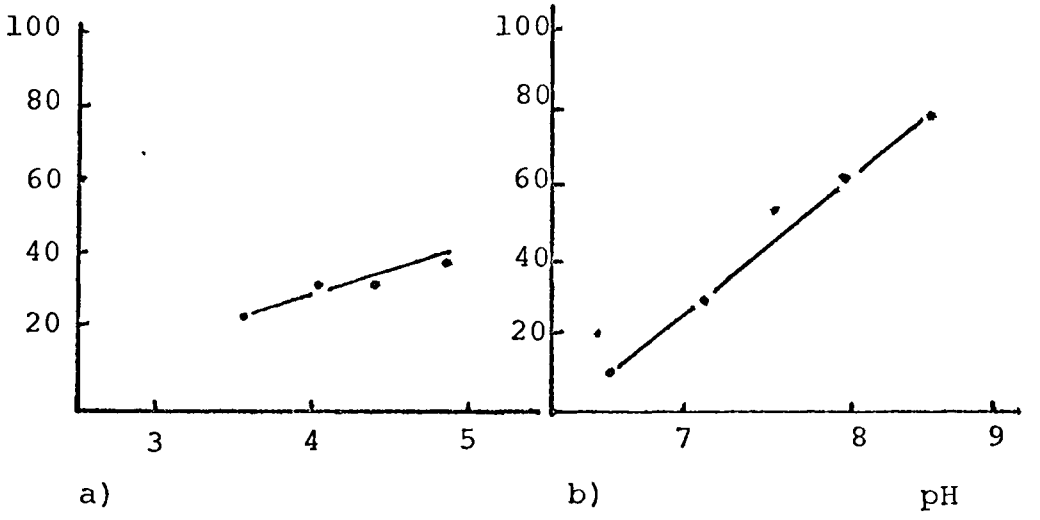

Figure 8. Fluorescence Dependence on pH.

(a) 20 mmol 4MU in 6 ml 0.167 M acetate buffer with quinine standard 1.
(b) 20 mmol 4MU in 6 ml 0.167 M acetate buffer with quinine standard 2.

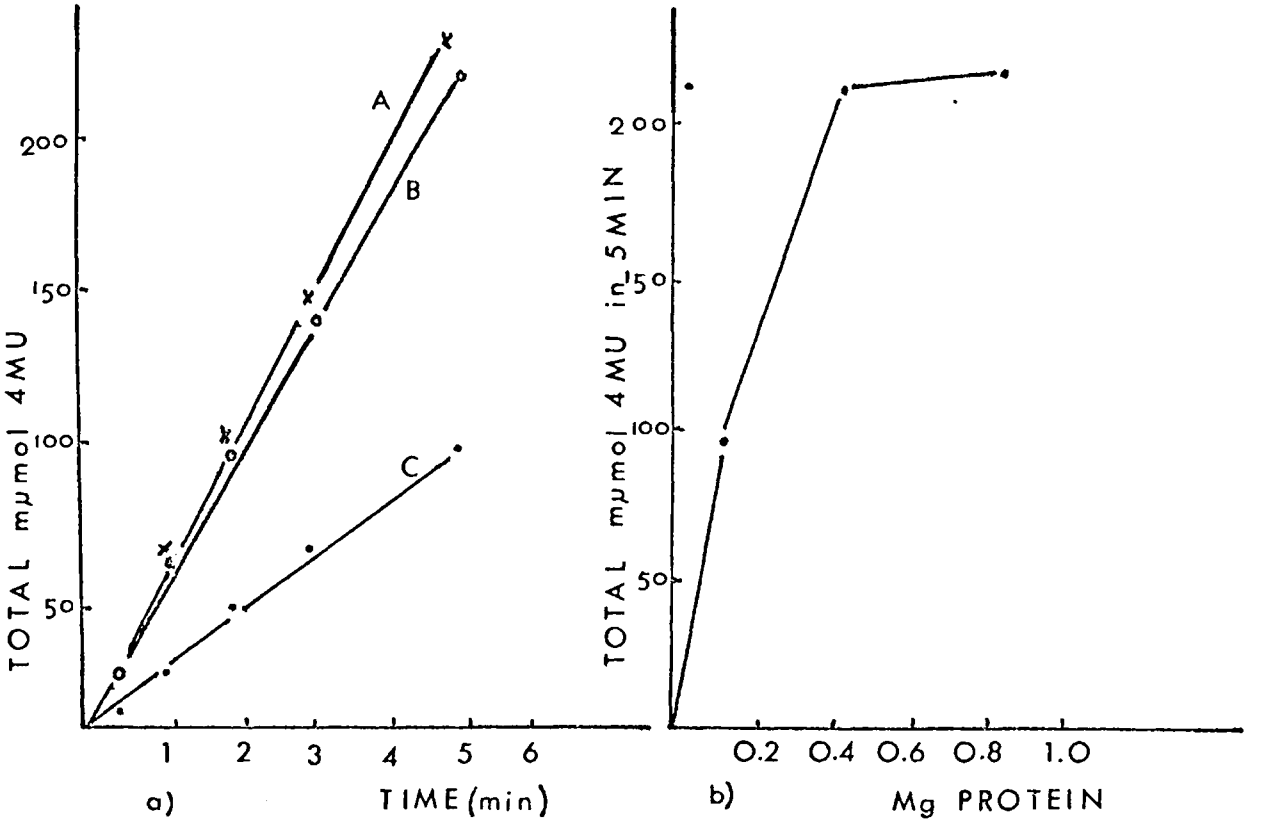

Figure 9. CBL vs Time in Fluorescent Assay.

(a) Each assay contained 600 mμmol 4MUC, 1 mmol acetate buffer, pH 4.4 in a reaction volume of 6 ml and varying amounts of protein as noted. A: 0.82 mg protein; B: 0.41 mg protein; C: 0.081 mg protein.
(b) Replotted data from 9(a).

concentration vs activity. Each assay contained 600 mμmol 4MUC (added as 0.1 ml of 6 x 10^{-3} M 4MUC in 6% dimethoxyethane), 1 mmol acetate buffer pH 4.4 and lipase (in TCE 7.9). The total reaction volume was 6 ml and each assay was followed for 5 minutes. Since lipase levels above 0.4 mg protein seemed to be saturating, the concentration of lipase for this assay was arbitrarily chosen as 0.08 mg protein or 0.25 mg crude lipase preparation.

Optimal activity of CBL was in a broad range of pH 3.6 to about 4.8 after which the rate dropped sharply to about 25% maximal activity at pH 5.0 (Figure 10). Since CBL is an acid lipase, it was surprising to find apparent hydrolysis occurring at alkaline pH values. (Curve C in Figure 11). Boiling the CBL or complete elimination from the assay still resulted in hydrolysis of 4MUC. It was found that rates with the boiled lipase added to the assay were somewhat higher than if the CBL was not added. The boiled CBL suspension contains Tris as buffer and this was suspected as acting as a catalyst in alkaline hydrolysis of the substrate. Catalysis by Tris seems to be borne out by the experiment represented in Figure 11 conducted at pH 7.5. Each assay contained 1 mmol acetate buffer pH 7.5 and 100 mμmol 4MUC. Reaction volume was 6 ml and followed

for 3 minutes. Curve A contained 2.5 mg boiled CBL (in 0.1 ml TCE 7.9), curve B had 0.1 ml TCE 7.9, but no boiled CBL, and curve C represents the absence of both TCE 7.9 and boiled CBL. Curve D represents the regular assay at pH 4.4 with boiled CBL indicating that the CBL is inactivated.

The optimal substrate concentration within the range of the fluorometer was from 0 to 600 mμmol 4MUC per 6 ml reaction volume. The stock solution of 1×10^{-3} M 4MUC was made up in 6% dimethoxyethane/ water, (v/v). Progress curves of 100 and 200 mμmol 4MUC/ 6 ml represented by curves A and B respectively in Figure 12(a) were linear with time, but a curious observation occurred when the 500 mμmol point was run. The fluorescence increased as expected then decreased considerably before resuming a linear response [curve C in Figure 12(a)]. The substrate aliquot for this assay contained 0.5 ml of solution in which there was effectively 0.03 ml of dimethoxyethane. When a total of 600 mμmol of substrate was added to the assay from a 6×10^{-3} M solution of 4MUC in 6% dimethoxyethane (i.e., 0.1 ml containing 0.006 ml dimethoxyethane), the phenomenon was not observed and the progress curve was linear without abnormality. [Curve D

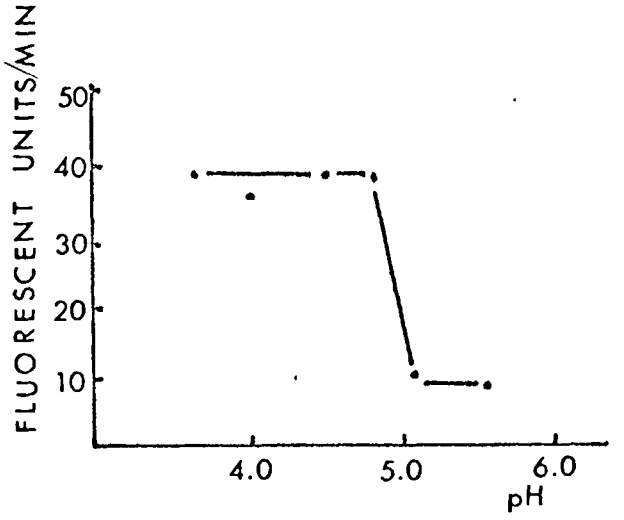

Figure 10. pH Profile in Fluorescent Assay.

Each assay contained 1 mmol acetate buffer at specified pH, 100 mμmol 4MUC and 0.081 mg protein in 6 ml total volume.

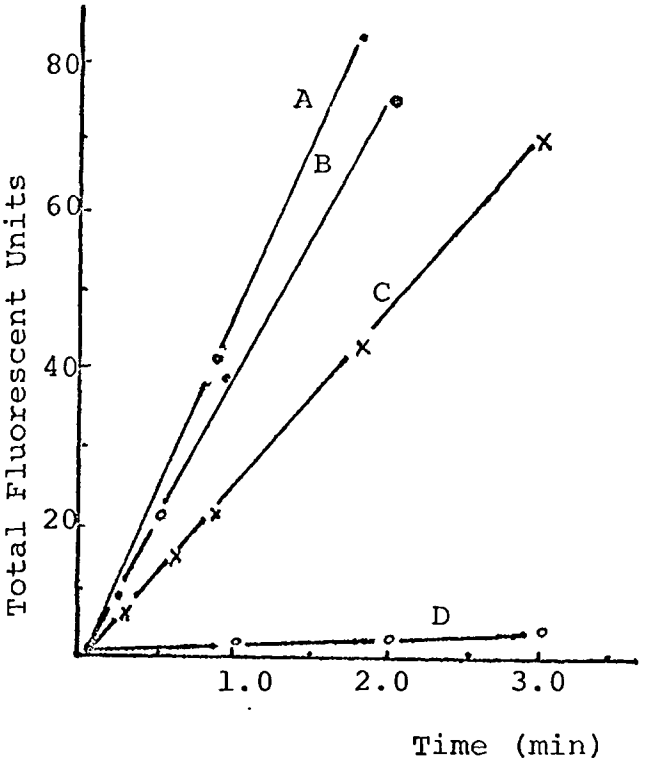

Figure 11. Autohydrolysis at Alkaline pH.

Each assay, except D, contained 1 mmol acetate buffer, pH 7.5, 100 mμmol 4MUC: Curve A had 0.08 mg boiled CBL in TCE 7.9; Curve B had no CBL but 0.01 ml TCE 7.9; Curve C had 0.08 mg CBL (boiled) in 1 mmol acetate buffer, pH 4.4.

in Figure 12(a)]. Neither was the effect seen if 0.03 ml of dimethoxyethane was added to the reaction mixture while the assay was in progress. Apparently the volume of dimethoxyethane over a threshold value in the presence of the lipase was responsible for the non-linear behavior. If the data in Figure 12(a) is replotted as substrate vs velocity (units/min) in Figure 12(b), it becomes obvious that the substrate concentrations used did not approach maximum velocity and that the subsequent working concentration of 300 mμmol per assay was not at saturation levels. However, this was necessitated because the available fluorometer was so sensitive that higher substrate concentrations would have required a smaller slit in the filters already at their minimum.

The stock solution of 4MUC was made up in 6% diethyl ether to determine if an effect similar to the one observed with dimethoxyethane could be seen. The results were negative, but the overall rates of reaction were somewhat lower. Finally, methylcellosolve (MC, 2-methoxyethanol) was also compared and found not to interfere at any concentration either by depression of the fluorescence or on the rate of reaction. Therefore a stock solution of the substrate was prepared in 6% MC for assay.

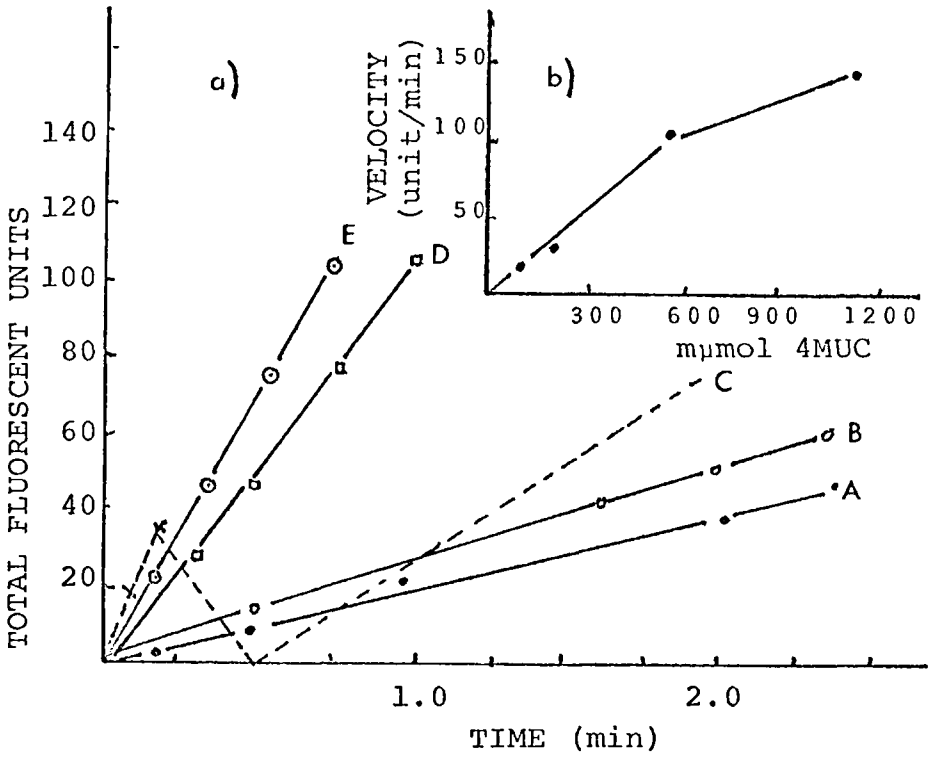

Figure 12. Effect of Dimethoxyethane.

Each assay contained 1 mmol acetate buffer pH 4.4 and 0.08 mg protein in 6 ml reaction volume. Curve A had 0.1 ml of 1 x 10^{-3} M 4MUC in 6% dimethoxyethane (100 mμmol); Curve B: 0.2 ml; Curve C: 0.5 ml; Curve D had 0.1 ml of 6 x 10^{-3} M 4MUC in 6% dimethoxyethane (600 mμmol); Curve E had 0.2 ml.

After optimal reaction conditions were determined for the fluorescent assay, a CBL* preparation was made by homogenization of 12.5 mg of CBL in 5 ml of TCE 7.9, adjusting to pH 5.2 with acetic acid and allowing the suspension to stand in ice for 20 minutes. The pH was then adjusted to 6.0 with NaOH and centrifuged. The resulting CBL* was then tested in the fluorescent assay with the results listed in Table 5.

Table 5. Activity of CBL* in Fluorescent Assay.

Each assay included 1 mmol acetate buffer, pH 4.4, 300 mμmol 4MUC, and 0.08 mg protein in 6 ml total volume.

ENZYME PREPARATION	ADDITION TO ASSAY	RATE (units/min)	CURVE (in Figure 12)
CBL	None	90	A
CBL(pH 5.2)	None	79	B
CBL*	None	75	C
CBL*	0.3 ml Fraction 3	51	D

The progress curves of the above reactions are shown in Figure 13. They indicate that the CBL* loses little activity after treatment at pH 5.2. Addition of Fraction 3 actually decreases the activity and is in direct contrast to the response of CBL* using cottonseed oil as substrate where the pH 5.2 treatment of CBL diminishes its activity. This experiment was repeated several times with similar results.

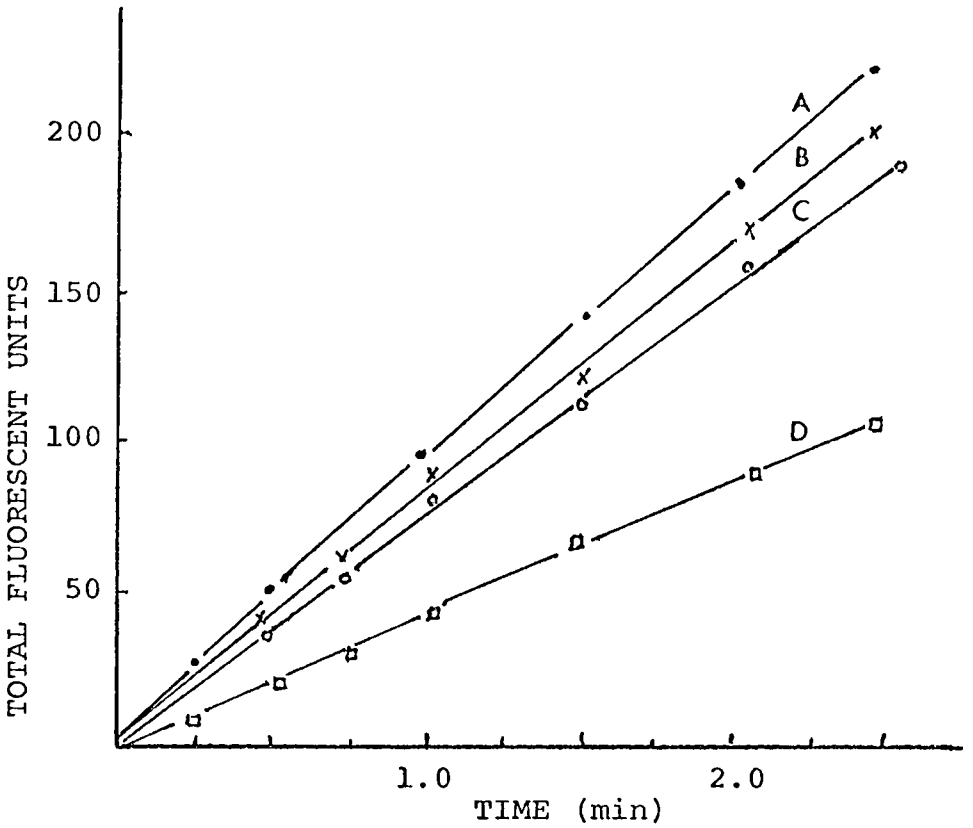

Figure 13. CBL* in Fluorescent Assay.

Reaction conditions as in Table 5.

In order to get some indication whether the enzyme active in the fluorescent assay was the same as that in the cottonseed oil experiments, cottonseed oil was added directly to the fluorescent assay mixture to determine if competition for substrate could be observe. A constant amount of cottonseed oil was added (0.01 ml) to increasing concentrations of 4MUC and compared to a similar assay that had no cottonseed oil added. The substrate was tested from 0 to 300 mμmol and indicated that rates of reaction were lower when the cottonseed oil was present. See Figure 14. Lineweaver-Burke plots in Figure 15 taken from the data in Figure 14 were inconclusive as to whether the inhibition by cottonseed oil was competitive or non-competitive, but favored the former slightly.

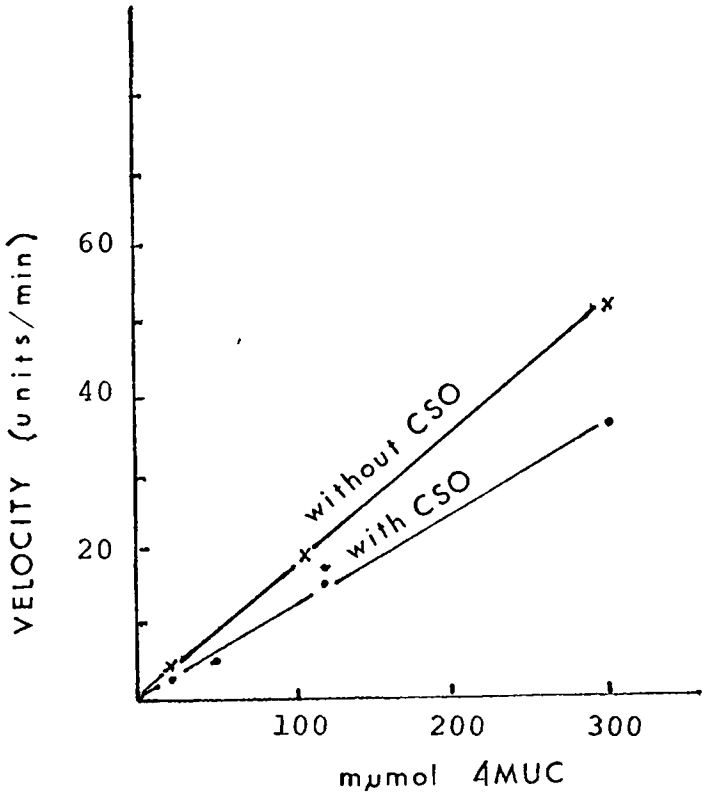

Figure 14. Inhibition of Fluorescent Assay by Cottonseed Oil.

Each assay contained 1 mmol acetate buffer, pH 4.4, 4MUC as indicated, 0.08 mg protein and 0.01 ml cottonseed oil in total reaction volume of 6 ml.

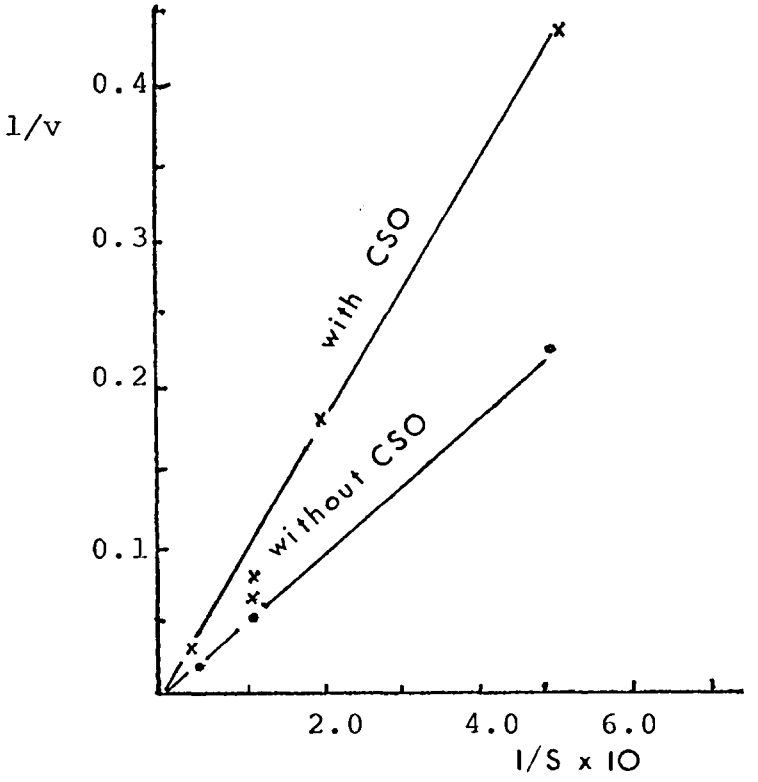

Figure 15. Lineweaver-Burke Plot.

This figure was constructed from data in Figure 14.

DISCUSSION

Similarities in properties, rates of lipolysis, response to pH 5.2 treatment and complete inhibition by PCMB seems to assure that the lipase studied here is the same as that reported in the literature.

The Tris soluble fraction (TS) apparently contains some moiety that increases the rate of lipolysis of CBL under certain conditions, but its effect is greatly negated by increasing the rate of agitation of the lipolytic reaction mixture or by lowering the pH of the lipase stock suspension from 7.9 to 4.4.

It is difficult to assess whether there is present a true activating species or whether the TS fraction is acting as a 'detergent-like' reagent by altering the properties of the micellular structure of the substrate. Passage of the TS fraction through a molecular sieve does not appear to concentrate a specific species but the experiment indicates that a family of species may activate the lipase. Increased rates of lipolysis are also evident for the lipase without the added TS fraction under conditions of increased agitation or lower pH of the stock suspension. Under the latter conditions, however, rates of lipolysis are independent of the method of agitation.

These experimental results suggest that there may be at least two factors which affect the rates of lipolysis. The first is that the lipase is sensitive to alkaline conditions and secondly, that it has a dependence on the size or physical properties of the micellular structure of the substrate.

The enzyme is most active if the stock suspension is at pH 4.4. Under these conditions, the lipase does not require increased agitation or added TS fraction for maximal activities. However, stock lipase suspensions in the alkaline range must undergo an alteration that decreases its activity. This decrease in activity of the partially inactivated enzyme can be overcome somewhat by increasing the surface area of the substrate/enzyme interface by increased agitation of the reaction mixture. The activity of the lipase under these conditions can also be increased by the addition of the TS fraction which may cause physical changes in the micellular structure of substrate. Effects similar to these have been reported in studies of activation of phospholipase A by addition of lipoproteins to micellular structures of lecithin (16).

The lipase is activated by sulfate and molybdate ions and this effect may extend to all divalent anions.

Anion activation of enzymatic reactions have been reported for several systems (17), but the mechanism here is unknown.

The fluorescent assay results indicate that the enzyme studied in that assay may not be the same as that in the cottonseed oil experiments since it does not become inactivated by the pH 5.2 treatment and removal of the supernatant. It apparently reacts with both 4MUC and cottonseed oil but at rates below those of the enzyme active in the two phase system. It is not possible to distinguish whether there are two enzymes reacting on each substrate independently or whether there is a single enzyme that reacts differently with the two substrates.

LITERATURE CITED

1. Green, J. R., Proc. Roy. Soc. London, 48B, 370 (1890).

2. Longenecker, H. E., Haley, D. E., J. Am. Chem. Soc., 57, 2019 (1935).

3. Bauman, E., Ullmann, E., and Tietz, N., Biochem. Zeit., 323, 489 (1953).

4. Altschul, A. M., Ory, R. L., and St. Angelo, A. J., Biochem. Prep., 10, 93 (1963).

5. Savary, P., Flanzy, J., and Desnuelle, P., Bull. Soc. Chim. Biol., 40, 637 (1958).

6. Weiss, H., Spiegel, H. E., and Titus, E., Nature, 183, 1393 (1959).

7. Spies, J. R., Coulson, E. J., J. Am. Chem. Soc., 65, 1720 (1943).

8. Layton, L. L., Mors, K. L., and De Eds, F., J. Am. Oil Chemist's Soc., 38, 76 (1961).

9. Ory, R. L., Yatsu, L. Y., and Kircher, H. W., Arch. Biochem. Biophys., 123, 255 (1968).

10. Ory, R. L., Barker, R. H., and Boudreaux, G. J., Biochemistry, 3, 2013 (1964).

11. Ory, R. L., Kircher, H. W., and Altschul, A. M., Biochim. Biophys. Acta, 147, 200 (1967).

12. Altschul, A. M., Ory, R. L., and St. Angelo, A. J., Fed.Proc. 18, 180 (1959).

13. Ory, R. L., St. Angelo, A. J., and Altschul, A. M., J. Lipid Research, 3, 99 (1962).

14. Jacks, T. J., and Kircher, H. W., Anal. Biochem., 21, 279 (1967).

15. Ory, R. L., St. Angelo, A. J., and Altschul, A.M., J. Lipid Research, 1, 208 (1960).

16. Attwood, D., Saunders, L., Gammack, D. B., De Haas, G. H., and van Deenan, L. L. M., Biochim. Biophys. Acta, 102, 302 (1965).

17. Dixon, M., and Webb, E. C., The Enzymes, 2nd Ed., Academic Press, New York (1964), pp. 443-449.