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PARTIAL PURIFICATION AND SOME PROPERTIES OF A LIPASE FROM *CORYNEBACTERIUM ACNES*

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SUMMARY

Because of its potential involvement in the etiologic events of the adolescent skin disease known as *Acne vulgaris*, a lipase (glycerol-ester hydrolase, EC 3.1.1.3) produced by *Corynebacterium acnes* was isolated and several of its properties described. The enzyme was purified approximately 200-fold from the culture supernatant by $(NH_4)_2SO_4$ fractionation and chromatography using Sephadex G-100. Polyacrylamide gel electrophoresis and isoelectric focusing demonstrated that the enzyme preparation was not homogeneous. The p*I* of this enzyme was pH 3.8. Double diffusion immunoprecipitation tests showed that the lipase preparation contained the major antigenic materials found in the whole bacterial cells. The enzyme displayed optimal activity at pH 7.5–9.0 with tributyrin being the best substrate found. Hydrolysis of triglycerides was shown to be completely random with respect to the position of glycerol in a triglyceride. The enzyme did not hydrolyze phospholipids or cholesterol linolenate.

INTRODUCTION

The etiological events which ultimately lead to an acne lesion are obscure. A current working hypothesis proposes that free fatty acids in the pilosebaceous follicles of human skin are the primary irritant in a series of events ultimately leading to an acne lesion[1–3]. However, it is known that the primary source of surface sebum, namely the sebaceous glands, produces a lipid mixture in which all fatty acids are esterified[4]. Yet, surface lipids contain significant amounts of free fatty acids, which have arisen from hydrolysis of triglycerides[5]. More significantly, comedones (sebaceous follicle plugs), which may lead to an inflamed acne lesion, demonstrate virtually complete hydrolysis of triglycerides to free fatty acids[6].

There is considerable evidence that enzymatic activity in the sebaceous follicles is responsible for the hydrolysis of sebum triglycerides. Esterases have been demonstrated histochemically in the sebaceous follicles[4] and lipolytic activity is demon-

Abbreviations: TEMED, *N,N,N',N'*-tetramethylethylenediamine.

strable in comedones[7]. Furthermore, the dominant microbial resident in human skin sebaceous follicles is *Corynebacterium acnes*[8,9] and this organism has been shown to be lipolytic[10–12]. Thus, it appears that these microbial lipases (glycerol-ester hydrolase, EC 3.1.1.3) hydrolyze sebaceous gland triglycerides to free fatty acids in the sebaceous follicle and the resulting free fatty acids in some way initiate the events which culminate in an acne lesion.

The postulated importance of *C. acnes* lipase has led us to isolate the enzyme and describe some of its properties.

EXPERIMENTAL

Materials

The following proteins were purchased from Nutritional Biochemical Co., Cleveland, Ohio: bovine serum albumin, catalase, ovalbumin, and bovine β-lactoglobulin. Yeast alcohol dehydrogenase was obtained from Calbiochem, Los Angeles, Calif., and lysozyme was purchased from Pentex Inc., Kankakee, Ill. Brain Heart Infusion Broth was purchased from Difco, Detroit, Mich.

$(NH_4)_2SO_4$ was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. *N,N,N',N'*-Tetramethylethylenediamine (TEMED), β-mercaptoethanol, sodium dodecyl sulfate (U.S.P.), acrylamide, methyl butyrate, and $(NH_4)_2S_2O_8$ were all purchased from Matheson, Coleman and Bell, Norwood, Ohio. Coomassie Brilliant Blue was obtained from Colab Laboratories, Chicago Heights, Ill. *N,N'*-Methylenebisacrylamide was purchased from Eastman Organic Chemicals, Rochester, N.Y.

Triglycerides were obtained as follows: triacetin, tripropionin, and tributyrin came from Matheson, Coleman and Bell; triundecanoin was acquired from Drew Chemical Company, Boonton, N.J.; tricaproin, trioctanoin, tridecanoin (practical grade), and tripalmitin were purchased from Eastman Organic Chemicals; trilaurin, trimyristin, and tristearin were prepared by R. G. Folzenlogen of these laboratories. All were used without further purification.

Cholesterol linolenate, 2,3-butanedioleate, and 1,4-butanedioleate were prepared from the appropriate fatty acid anhydrides and alcohols. Similarly, 2,3-di-*O*-oleoyl-glyceryl-1-palmitate and 1,3-di-*O*-oleoyl-glyceryl-2-palmitate were prepared from the appropriate diglyceride and fatty acid anhydride[13]. Triolein was purified from olive oil and was better than 98% pure by gas–liquid chromatography and thin-layer chromatography.

Measurement of enzymatic activity

Lipase activity was determined by measuring the rate of hydrolysis of a triglyceride emulsion by potentiometric titration, using a pH-stat (Radiometer pH Meter 26 connected to a Titrator 11 and Titrigraph SBR 2c, obtained from The London Co., Westlake, Ohio). One unit of enzymatic activity is defined as 1 μmole of acid produced/min at 25°. Substrate was prepared by emulsifying 175 μmoles of triglyceride with 15 ml of 5 mM $CaCl_2$ and 0.2 M NaCl or 1.0 M NaCl. The latter concentration of NaCl was used in all experiments involving triglycerides of longer chain length than tributyrin in order to insure complete titration of fatty acid[14]. Emulsification was effected by use of a Branson Sonifer (Heat Systems Co., Melville, N.Y.) using the microtip for 15 sec at 8 A directly in the reaction vessel. Enzyme

(25–500 μl, 0.05–1.0 unit) was then added to freshly prepared substrate solution, the pH was rapidly adjusted to pH 8.0, and the rate of acid production was measured by monitoring the continuous consumption of 5 mM KOH. The stirring of the vessel was at maximum setting and the reaction vessel was continuously flushed with CO_2-free N_2. The slope of the pH-stat data was calculated using the linear portion of the curve and converted to microequivalents of KOH consumed/min. The rate obtained was shown to be enzyme-limited and was linear with time for up to 20 min.

Preparation of the enzyme

C. acnes strains were obtained from J. G. Voss of this laboratory. Stock cultures were maintained as previously described[15]. *C. acnes* D-34 was the strain selected for isolation of *C. acnes* lipase. Enzyme was obtained after growth of the organisms 4–5 days at 37° in an atmosphere of N_2–CO_2 (95:5, v/v) in 1000 ml Brain Heart Infusion Broth fortified with 1% glucose. After this incubation period (stationary growth phase), the bacterial cells were centrifuged and discarded, and the culture supernatant was stored at 4°. Less than 5% of total lipolytic activity was associated with the bacterial cells. Under these conditions, enzymatic activity is stable for a period of several months.

$(NH_4)_2SO_4$ precipitation was performed on the culture supernatant. Using the nomograph of DIJESO[16], enough $(NH_4)_2SO_4$ was added to 600 ml of culture supernatant (kept in an ice bath) such that the solution was 60% saturated in $(NH_4)_2SO_4$. The pH was then adjusted to pH 6.2 using NH_4OH. After stirring for 2–3 h at 4°, the solution was centrifuged and the supernatant discarded. The precipitated material was dissolved in a minimal amount of water (25–30 ml) and dialyzed against 4 l of 0.1 M NaCl overnight in the cold. This material was stable for several weeks in the cold and represented $(NH_4)_2SO_4$-fractionated material.

Sephadex G-100 chromatography was then performed on the $(NH_4)_2SO_4$-fractionated material using a 2.5 cm × 40 cm column. Routinely 10 ml of sample was applied and the column was eluted with 0.1 M NaCl, 0.02 M acetate, pH 5.2. Fractions of 4.0 ml were collected, protein content estimated by measuring absorbance at 280 nm, and lipase activity assayed. Those fractions containing high lipolytic activity were combined and stored at −20°.

pH optimum studies

Assays were set up as described with the pH stat set to the indicated endpoint, using tributyrin as substrate. For pH values of pH 6.0 and below, a standard butyric acid solution was prepared and titrated to the given pH as endpoint and then compared to the titration value at pH 8.0. This enabled a correction to be made for the observed enzymatic rates at pH values of pH 6.0 and below to allow for the incomplete titration of butyric acid at the given pH.

Isoelectric focusing

An electrolysis column of 110 ml capacity and various synthetic carrier ampholytes were purchased from LKB Instruments, Rockville, Md. The column was cooled throughout the experiment with circulating water at 4°. The LKB gradient mixing device (110 ml) was used to apply samples. The samples had been thoroughly dialyzed against water. The instructions in the LKB Electrofocusing Equipment Instructions

Manual were followed exactly. Ampholyte concentrations of 1% were used. After all liquid had been introduced to the column and the central tube had been opened, electrolysis was initiated by applying 400 V of electric tension. The voltage was applied until a constant current of 2 mA was obtained (usually 12–15 h), keeping the temperature at 4° throughout. The central tube of the column was then closed and the contents of the column were drained slowly by gravity feed through tubing into a fraction collector. Approximately 3 ml-fractions were collected, absorbance at 280 nm was measured, pH was determined using the Radiometer pH Meter 26, and aliquots were assayed for lipolytic activity.

Preparation of C. acnes antibody

Rabbit antisera to the 2 groups of *C. acnes* were kindly provided by J. G. Voss of this laboratory. Antisera preparation and double-diffusion precipitin tests were carried out as previously described[15].

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described by WEBER AND OSBORN[17]. The following proteins were used as standards: bovine serum albumin, catalase, ovalbumin, yeast alcohol dehydrogenase, β-lactoglobulin, and lysozyme. All protein standards were prepared by dissolving the protein directly into 0.01 M phosphate, pH 7, 1% sodium dodecyl sulfate, and 1% β-mercaptoethanol and incubating for 3 days at 37°. Lipase preparations were dialyzed in this buffer for 3 days at 37°. All samples were then dialyzed for several hours at room temperature against 0.01 M phosphate, pH 7, 0.1% sodium-dodecyl sulfate, and 0.1% β-mercaptoethanol.

Gel buffer and acrylamide–methylenebisacrylamide solutions were prepared as described[17]. All experiments were performed using gels with 7 1/2% acrylamide and "normal" amount of crosslinker (methylenebisacrylamide). Gels were formed in tubes of 10 cm length and an internal diameter of 6 mm. Samples were applied and electrophoretic runs performed at a constant current of 8 mA per tube using the Canalco apparatus (Canalco, Bethesda, Md.). After the marker dye (bromphenol blue) had migrated to near the bottom of the gel, electrophoresis was terminated, gels were removed from the glass tubes, and placed into the staining solution which contained Coomassie Brilliant Blue. After staining overnight, gels were then placed into destaining solution for several hours and then destained electrophoretically using the Canalco destaining apparatus. Protein bands were located and the distance of the leading edge of the band from the origin was measured. Mobilities were calculated as described[17].

Substrate specificity studies

Qualitative measure of enzymatic activity was obtained by incubating an emulsion of the given substrate in 10 ml of 0.04 M Tris, 0.2 M NaCl, pH 8, along with purified lipase preparations. After stirring the reaction for several hours at room temperature, 1 ml of 0.5 M H_2SO_4 was added to the reaction. This material was then extracted 3 × 50 ml with ether, the combined ether extractions were water-washed, dried with anhydrous Na_2SO_4, and evaporated. This material was then analyzed by

thin-layer chromatography and compared with no-enzyme control to check for possible activity by *C. acnes* lipase.

Alternatively, 175 μmoles of a given substrate were emulsified with the $CaCl_2$–NaCl solution and assayed in the pH-stat as previously described.

The action of *C. acnes* lipase on the synthetic triglycerides 2,3-di-*O*-oleoyl-glyceryl-1-palmitate and 1,3-di-*O*-oleoyl-glyceryl-2-palmitate was examined in detail. To 15 ml of 5 mM $CaCl_2$ and 1.0 M NaCl was added 115 μmoles of 2,3-di-*O*-oleoyl-glyceryl-1-palmitate or 1,3-di-*O*-oleoyl-glyceryl-2-palmitate and this mixture was emulsified by sonication. Purified lipase was added and digestion was monitored using the pH-stat. The reaction was maintained at pH 8 using 0.04 M KOH and terminated after 55% hydrolysis (3 h). Each reaction was acidified with 1 ml of 0.5 M H_2SO_4 and then extracted 3 × 50 ml with ether. The ether extracts were water-washed, dried with anhydrous Na_2SO_4, and evaporated to dryness with N_2 and weighed. Recovery of original material was 80% by weight (glycerol, which would be in the water layer, was not estimated). Thin-layer chromatography using silicic acid (hexane–isopropyl ether–acetic acid, 70:30:1, by vol.) was then performed on the digested material after redissolving in 10 ml of redistilled hexane.

The bulk of the sample was applied to Florisil columns[18] in order to fractionate released fatty acids from esterified fatty acids. Florisil (purchased from the Floridin Co., Berkeley Springs, W. Va.) was deactivated by the addition of 7 ml of water to 100 g Florisil. This material (10 g) was slurried with redistilled hexane and packed into a column. The digested sample in hexane was then applied. The column was then successively eluted with 150 ml of 50% ether in hexane (Fraction 1, triglycerides, and diglycerides), 85 ml of 2% methanol in ether (Fraction 2, monoglycerides), and finally 4% acetic acid in ether (Fraction 3, free fatty acids). Analyses by thin-layer chromatography showed that separations were complete. Each fraction was evaporated to dryness, dissolved in 2% H_2SO_4 in methanol, and refluxed for 90 min in order to make methyl esters for gas–liquid chromatography analysis. After refluxing, 60 ml water was added and the samples were extracted with 3 × 50 ml ether, the combined ether extracts were water washed, dried with anhydrous Na_2SO_4, and evaporated. Each sample was then dissolved in a small volume of hexane for gas–liquid chromatography analysis.

Gas–liquid chromatography was performed using a Hewlett Packard Model 720 unit containing a 10 ft × 0.25 inch stainless steel column packed with 15% DEGS on 60–70 mesh Anakrom ABS. All runs were done at isothermal operation with the column at 190°, the injection port at 300°, and the thermal conductivity detector at 300°, with a flow rate of 70 ml He/min (standard temperature and pressure).

RESULTS AND DISCUSSION

Purification of the enzyme

Table I demonstrates the amount of purification obtained thus far with *C. acnes* lipase. Purifications of 175–200 fold are routinely obtained by using this scheme. Fig. 1 shows the elution pattern obtained when the $(NH_4)_2SO_4$-fractionated material is placed over a Sephadex G-100 column. It is evident that all lipolytic activity is in the first protein peak, which is material that has been excluded by the column (determined by measuring column void volume with Blue Dextran). We have found

TABLE I

PURIFICATION OF *C. acnes* LIPASE

Fraction	*Vol. (ml)*	*Activity (units/ml tributyrin substrate)*	$A_{280\ nm}$	*Units/ml per* $A_{280\ nm}$	*Total units*	*Recovery (%)*	*Activity ratio tributyrin/triolein*	*Purification factor*
Culture supernatant	600	5.0	24.0	0.2	3000	—	8.1	—
0–60% $(NH_4)_2SO_4$ precipitate	40	69.0	16.5	4.2	2760	92	8.7	21
Sephadex G-100 chromatography (repeat 4 times)	100	17.0	0.43	39.5	1700	55	9.6	198

that this enzyme is also excluded by Sephadex G-200 but no further purification is obtained. Thus it appears to have a very large molecular weight and/or is complexed with material of high molecular weight.

Further purification of this material has not thus far been attempted, although the isoelectric focusing and disc gel electrophoresis data presented below show that additional purification is possible. However, when compared on an equivalent specific activity basis (μequiv free fatty acids produced/min per mg protein) this preparation of *C. acnes* lipase is more active than the lipase preparation from *Pseudomonas fragi*[19] and the lipase preparation from *Propionibacterium shermanii*[20].

Extent of lipase purification

Fig. 2 illustrates the isoelectric focusing profile obtained with *C. acnes* lipase using a pH gradient of pH 3–6. While only one protein peak was obtained, it is apparent that lipase activity does not completely coincide with protein indicating the presence of other material. The p*I* of *C. acnes* lipase was estimated to be pH 3.8.

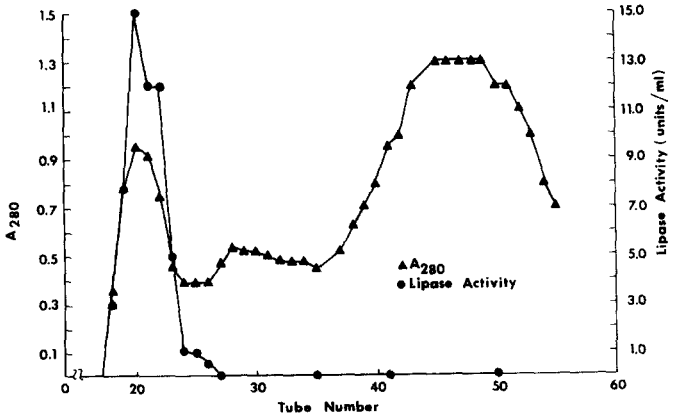


Fig. 1. Elution of $(NH_4)_2SO_4$-fractionated material from Sephadex G-100 column. Conditions are described in EXPERIMENTAL.

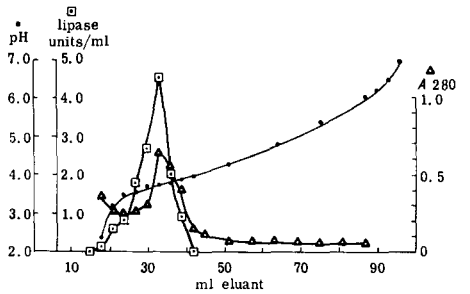


Fig. 2. Isoelectric focusing of *C. acnes* lipase after Sephadex chromatography. Conditions are described in EXPERIMENTAL.

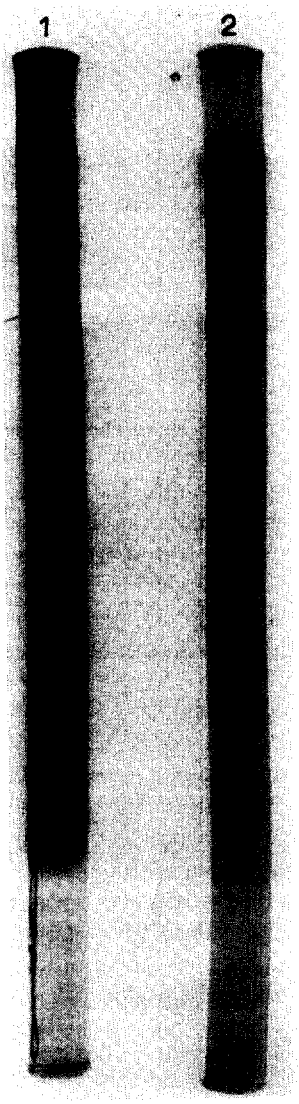
However, most of the enzymatic activity was lost under the conditions of the experiment.

It was of interest to develop an electrophoretic system to monitor the various purification techniques. Lipase activity was routinely assayed by incubating the inert electrophoretic support (*e.g.* cellulose acetate or polyacrylamide gel) in a tributyrin emulsion in agar. Zones of clearing of the emulsion indicated lipase activity. Several parameters were varied using polyacrylamide gel electrophoresis, *e.g.* pH, percent gel concentration, amount of crosslinking, but under no conditions could mobility of *C. acnes* lipase be demonstrated.

Thus use was made of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis system described by WEBER AND OSBORN[17]. Unfortunately the high concentrations of sodium dodecyl sulfate and β-mercaptoethanol employed irreversibly denature *C. acnes* lipase. However, it was possible to observe mobility of the material in this system, as Fig. 3 demonstrates. Fig. 4 shows that by using a series of protein standards it was possible to correlate protein mobility in this system with molecular weight. Note that while the Sephadex-purified material in Fig. 3 displays several bands, the two major bands have the lowest mobility. The position of these bands relative to standard proteins is shown by the arrows in Fig. 4. Even under conditions of great denaturation these materials have a very high molecular weight. It is difficult to estimate molecular weights of this magnitude by this technique because of the lack of suitable standards.

Antigenicity of C. acnes lipase

Rabbit antisera were prepared to washed, whole-cell suspensions of two strains of *C. acnes*, 6919 and D-34. These strains are the archetypes of *C. acnes* Group I and *C. acnes* Group II, respectively, and are described in more detail elsewhere[15]. One of the ways of differentiating strains of *C. acnes* into Group I or II is by agar diffusion tests with a homologous and heterologous antiserum. It was of interest to determine the cross-reactivity of this *C. acnes* lipase preparation (from strain D-34) with *C. acnes* D-34 antiserum and with antiserum to a Group I organism, *C. acnes* 6919. Results obtained when *C. acnes* lipase preparations at each stage of purification were allowed to interact with *C. acnes* antisera are displayed in Fig. 5. It is evident that even the Sephadex-purified material reacts strongly with the *C. acnes* D-34 antiserum to give a reaction of identity, although a spur is present (Fig. 5a). Note also that there are



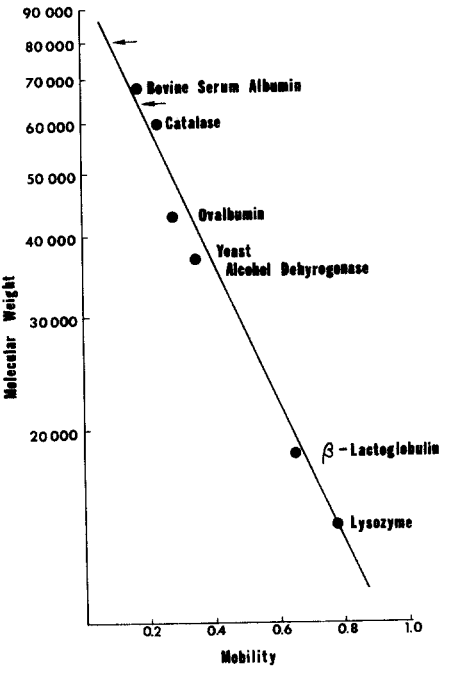


Fig. 3. Polyacrylamide gel electrophoresis of $(NH_4)_2SO_4$-fractionated lipase (left gel) and Sephadex-purified lipase (right gel) using the sodium dodecyl sulfate system. Applied 500 μg of protein to left gel and 40 μg protein to right gel. Mobility was from top to bottom.

Fig. 4. Semilog plot of molecular weight *versus* mobility in the sodium dodecyl sulfate –polyacrylamide gel electrophoresis system for the indicated standard proteins. Arrows indicate the mobilities of the two major bands observed in the electrophoresis of *C. acnes* lipase (*cf.* Fig. 3).

at least two precipitin bands in the Sephadex-purified lipase interaction with the antiserum, indicating probable heterogeneity in the lipase preparation. In contrast, all lipase preparations give a very weak reaction with the *C. acnes* 6919 antiserum (Fig. 5b). The reaction appears weakest in the Sephadex-purified material, indicating that partial purification of the enzyme results in removal of antigenic material com-

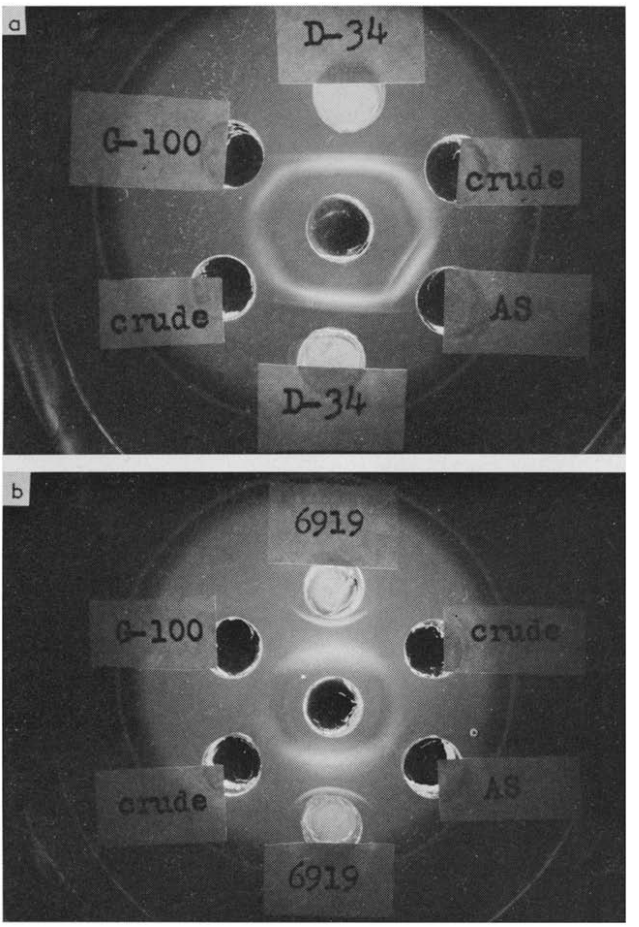


Fig. 5. Agar-diffusion precipitation tests. (a) Center well contains rabbit antiserum to *C. acnes* D-34 (Group II), outer wells contain cell suspension of *C. acnes* D-34 (labelled as "D-34"), culture supernatant of *C. acnes* D-34 ("crude"), $(NH_4)_2SO_4$-fractionated *C. acnes* lipase ("AS"), and Sephadex-purified lipase ("G-100"). (b) Center well contains rabbit antiserum to *C. acnes* 6919 (Group I), outer wells same as (a) except for wells containing *C. acnes* 6919 cell suspensions. Both plates reacted for 48 h at room temperature.

mon to both *C. acnes* Groups I and II. It should be pointed out that although *C. acnes* 6919 (and most Group I strains in general) is weakly lipolytic when growth is observed on an agar plate containing emulsified triglyceride (J. A. WHITESIDE AND J. G. VOSS, personal communication), we have never been able to demonstrate lipolytic activity in a static culture of this organism using the pH-stat assay. However, other Group I strains display enough lipolytic activity to measure by the pH-stat method.

Fig. 6 demonstrates the interaction of *C. acnes* lipase preparations with rabbit antiserum to the homologous strain, *C. acnes* D-34 (Group II), which was absorbed with a cell suspension of *C. acnes* 6919 (Group I), thus eliminating all cross-reacting antibodies in the rabbit antiserum preparation. The Sephadex-purified lipase material displays only one sharp band of precipitation with the absorbed antiserum after 20 h (Fig. 6a) and 44 h (Fig. 6b) of reaction, in contrast to the reactions displayed by the

whole cell suspension of *C. acnes* D-34 and the crude lipase preparation ($(NH_4)_2SO_4$-precipitated material). Notice that the purified lipase preparation does not contain several of the precipitation bands evident in the other reactions. However, the single precipitation band observed with the purified lipase material does form a reaction of identity with the major band of the whole cell-antiserum interaction. Removal of

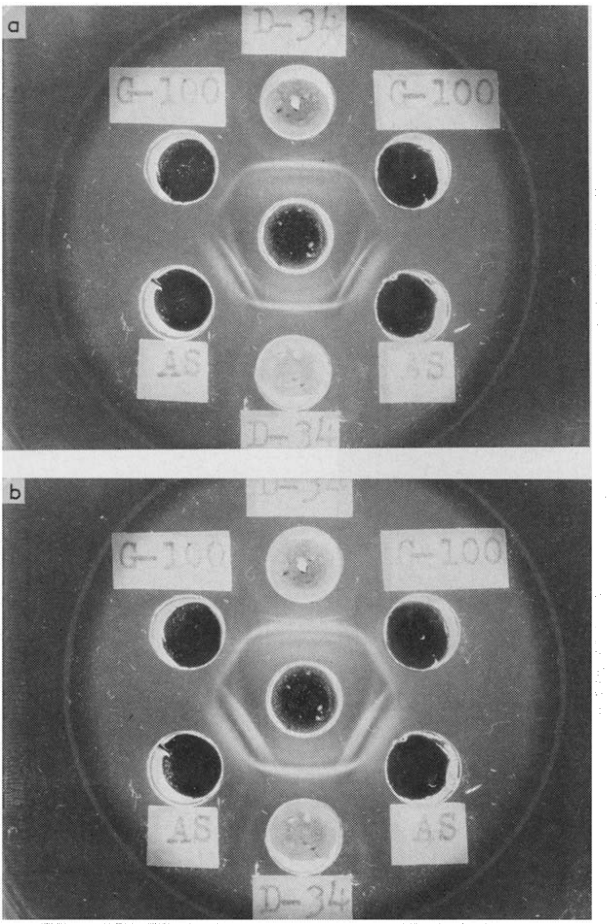


Fig. 6. Agar-diffusion precipitation tests. (a) Center well contains rabbit antiserum to *C. acnes* D-34 (Group II) absorbed with *C. acnes* 6919 (Group I) cells, outer wells contain *C. acnes* D-34 cell suspension ("D-34"), $(NH_4)_2SO_4$-fractionated lipase ("AS"), and Sephadex-purified lipase ("G-100"). (b) Same plate, 44 h reaction time.

cross-reacting antibodies by absorption of this antiserum with *C. acnes* 6919 has considerably sharpened the bands of interaction, perhaps by removing the weakly cross-reacting antibodies. At any rate this preparation of *C. acnes* lipase does contain the major antigenic component(s) found in the whole cells. Reaction of this antiserum with a homogeneous preparation of the lipase would be necessary to determine if the lipase itself is this major antigenic component.

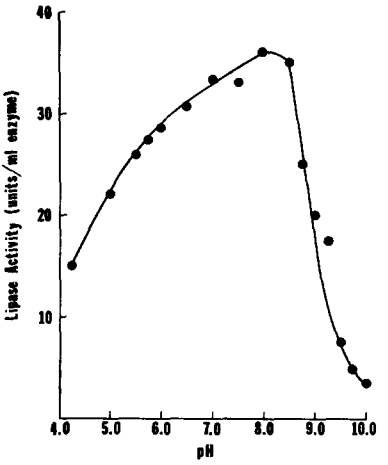


Fig. 7. Effect of pH on *C. acnes* lipase activity. See text for conditions.

pH optimum

This enzyme has optimal activity at pH 7.5–8.0, as illustrated in Fig. 7. In addition, the enzyme displays considerable activity at pH values below pH 7. This fact may be of significance *in vivo;* the large amounts of free fatty acids found in surface sebum[5] and comedones[6] may indicate an environment of low pH for the bacteria and the enzyme *in vivo*. These studies of the effect of pH on activity indicate that the lipolytic activity is not affected greatly by lower pH values.

Substrate specificity

The possibility of fatty acid chain length specificity by *C. acnes* lipase was investigated using a series of synthetic triglycerides, each with only one type of fatty acid esterified to the three glycerol hydroxyls. The results obtained upon examination

TABLE II

LIPASE ACTIVITY ON TRIGLYCERIDES

175 μmoles substrate, pH 8.0, 25°.

Triglyceride	*Enzyme activity (units/ml)*
C_2	4.5
C_3	18.0
C_4	18.0
C_6	9.0
C_8	6.0
C_{10}	4.3
C_{11}	4.2
C_{12}	3.1
C_{14}	1.0
C_{16}	0.1
C_{18}	0
$C_{18:1}$	1.5

TABLE III

LIPASE DIGESTION OF 2,3-DI-*O*-OLEOYL-GLYCERYL-1-PALMITATE AND 1,3-DI-*O*-OLEOYL-GLYCERYL-2-PALMITATE

About 55% digestion.

		Mole %	
		Palmitate	*Oleate*
2,3-Di-*O*-oleoyl-glyceryl-1-palmitate	Original	32.7	67.3
	Triglycerides + diglycerides	22.0	78.0
	Free fatty acids	29.6	70.4
1,3-Di-*O*-oleoyl-glyceryl-2-palmitate	Original	33.8	66.2
	Triglycerides + diglycerides	50.0	50.0
	Free fatty acids	20.9	79.1

of the series of triglycerides of increasing fatty acid chain length beginning with triacetin up to a fatty acid chain length of 18 carbons (tristearin and triolein) is shown in Table II. Triglycerides displaying best activity were the shorter chain length triglycerides, tripropionin, and tributyrin. Enzyme activity appears to decrease with increasing fatty acid chain length of each triglyceride. However, the data is no doubt complicated by the fact that triglycerides of higher molecular weight than tridecanoin are solids at room temperature (except the unsaturated triglyceride triolein). The reduction in lipase activity may be due to the increasing melting point of the substrate. Comparison of the enzyme activity when tristearin and triolein are substrates speaks to this point. Although the fatty acid chain lengths are identical, the unsaturation in oleic acid lowers the melting point of triolein so that it is a liquid at room temperature. *C. acnes* lipase is active against this triglyceride but does not hydrolyze tristearin (within limits of detection in this experiment).

In order to avoid the effect of substrate melting point on *C. acnes* lipase activity, and at the same time gain insight into the position(s) of the glyceride molecule hydrolyzed by the enzyme, the hydrolysis of two defined triglycerides by this enzyme was examined. The two triglycerides were 2,3-di-*O*-oleoyl-glyceryl-1-palmitate and 1,3-di-*O*-oleoyl-glyceryl-2-palmitate. Both triglycerides have similar melting points and are liquids at 25°. Table III displays the palmitate and oleate content of the lipid fractions, as determined by gas–liquid chromatography of the methyl esters, after each triglyceride was digested separately by the enzyme. Triglycerides and di-

TABLE IV

RELATIVE LIPASE ACTIVITY ON A VARIETY OF SUBSTRATES

Substrate	*Activity*
Tributyrin	24.0
Triolein	4.4
1,3-Diolein	3.1
2-Monoolein	0.2
1,4-Butanedioleate	0.75
2,3-Butanedioleate	0.35

glycerides were combined in the fractionation; too little monoglyceride was formed to isolate (less than 1% by thin-layer chromatography). It is evident that the free fatty acids released from 2,3-di-*O*-oleoyl-glyceryl-1-palmitate are identical in composition to the original triglyceride, suggesting a random hydrolytic attack of all three positions on the starting material. Free fatty acids released by *C. acnes* lipase from 1,3-di-*O*-oleoyl-glyceryl-2-palmitate differ somewhat from the starting material in the digestion in that there is less palmitate (less hydrolysis of the 2-position in the original triglyceride). However, the level of palmitate in the free fatty acids is high enough to again suggest quite random attack on triglycerides by this enzyme. It should again be emphasized that thin-layer chromatography analysis of a triglyceride digestion by this enzyme routinely shows very little diglyceride and almost no monoglyceride after significant hydrolysis has occurred ($> 50\%$).

The results shown in Table IV demonstrate that *C. acnes* lipase does indeed readily hydrolyze secondary esters, *e.g.* the 2-position in a triglyceride. Note that the rate with 1,3-diolein is similar to that with triolein. Although the rate displayed against 2-monoolein is very low, this may be due to the two free hydroxyl groups of the glycerol moiety causing an inhibition of the lipolysis (R. A. VOLPENHEIN, personal communication, *cf.* ref. 21). Stronger evidence is given by the relative rates of hydrolysis of the two diesters of butanediol, 1,4-butanedioleate, and 2,3-butanedioleate. The first substrate has two primary ester groups but is hydrolyzed at only twice the rate of the second substrate which has two secondary ester groups. Thus this lipase does hydrolyze secondary ester linkages at a sufficient rate compared to primary esters to allow one to explain the complete hydrolysis of 2,3-di-*O*-oleoyl-glyceryl-1-palmitate and 1,3-di-*O*-oleoyl-glyceryl-2-palmitate to glycerol and free fatty acids.

The substrate specificity of this enzyme correlates well with the analysis of lipids found on the skin surface. While there are large amounts of free fatty acids and triglycerides in sebum, there are only small amounts of diglycerides and very little monoglycerides detectable[22]. The inverse relationship of free fatty acids and triglycerides suggests that hydrolysis of triglycerides is the source of free fatty acids in sebum. The random hydrolysis of triglycerides to glycerol and free fatty acids, with little buildup of di- and monoglycerides, which we have observed with *C. acnes* lipase would fit into this pattern.

Long term incubations (3 h) of high levels of *C. acnes* lipase (20 units of enzyme activity, based on tributyrin) followed by thin-layer chromatography analysis failed to show any release of free fatty acids from cholesterol linolenate, lecithin, lysolecithin, or phosphatidyl ethanolamine. Thus this enzyme apparently cannot attack sterol esters, a component of skin surface lipids.

The specificity of *C. acnes* lipase appears to be similar to that of the lipase from castor bean[23] and the lipases from *Staphylococcus aureus* and *Aspergillus flavus*[24]. There is some evidence that the castor bean enzyme is able to hydrolyze esters at all of the positions of glycerol in a triglyceride by catalyzing the isomerization of a 1,2-diglyceride to a 1,3-diglyceride and then hydrolyzing the primary esters of the resulting latter diglyceride. Although no direct evidence is presented in this communication which has direct bearing on this point, we have observed both 1,2- and 1,3-diglycerides in the digestion products formed upon hydrolysis of a triglyceride by *C. acnes* lipase.

The survey of microbial lipase specificity by ALFORD *et al.*[24] demonstrated that

three main types of specificity occur. Lipases of the first type attack primarily the 1-position of a triglyceride in a manner analogous to that displayed by pancreatic lipase. The lipases from *Staphylococcus aureus* and *Aspergillus flavus* comprised the group displaying the second type of specificity, namely, the capacity to cleave all three positions of a triglyceride. Specificity for the fatty acid portion of a triglyceride was displayed by the lipase from *Geotrichum candidum* and was the only example of this third class of specificity. It would appear that the specificity displayed by *C. acnes* lipase would place this enzyme in the second category of the above. More data must be obtained to determine if in addition this enzyme displays any fatty acid specificity.

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