PARTIAL PURIFICATION AND SOME PROPERTIES OF LIPASE FROM PSEUDOMONAS AERUGINOSA

THESIS

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By

Linda Kay Morrison, B. S.
Denton, Texas
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Purification of lipase from Pseudomonas aeruginosa (from both a washed cell suspension and crude culture supernatant as the enzyme source) was performed utilizing affinity chromatography. Affinity chromatography was carried out using n-dodecylamine bound to Sepharose 4B. Chromatography of the concentrated crude culture supernatant resulted in a 65 to 95 fold purification with 5.8% recovery. Washed cells collected from a ten hour culture suspended in water also produced enzyme. Activity of the washed cell suspension supernatant was found to be 4.5 fold higher than the activity of the culture supernatant. A thirty percent recovery was obtained using the washed cell suspension supernatant. The washed cell suspension provides a cleaner preparation for use with the dodecylamine-agarose chromatography in purifying the enzyme.

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CHAPTER I

INTRODUCTION

Lipases have been isolated and purified from animals, and, to a more limited extent, from microorganisms (32). The properties of the bacterial lipases have not been fully elucidated despite the wide diversity of literature that has accumulated. The diversity of these data originates, in part, from the use of crude enzyme preparations and from the variety of substrates, assay conditions, and methods used to determine lipase activity. Lipases form a rather indefinite group of the esterases. Lipases are distinguished from other esterases in that they hydrolyse emulsified esters of glycerol, whereas esterases hydrolyse dissolved substrates. The choice of any substrate is arbitrary, since little is known of their natural substrates or their physiological role in the metabolism of bacteria (18).

Much of the interest in bacterial lipases has been taxonomic. In one study it was reported that of about 100 bacterial strains tested, nearly all Gram-positive and Gram-negative organisms produced lipase, although there was considerable variation in lipolytic activity between genera and even between strains of a species (18). Organisms producing lipase include, but are not limited to, members of the genera Micrococcus, Pseudomonas, Acinetobacter, Staphylococcus, and Corynebacterium (5, 6, 18, 30, 32). The lipases from some of these organisms have been studied to investigate their role in pathogenicity.

Studies performed in other laboratories (7, 21, 22, 23) have indicated that the extent to which Pseudomonas aeruginosa can establish an infection depends on the pathogenic properties of the particular strain as well as the degree of susceptibility of the host. Extracellular products with possible significance in the pathologic process include proteases, collagenases, a hemolysin, an elastase, and a lipase (25, 29). The lipase has not received much attention and its contribution to virulence of this species is not clear.

Methods used in purification of lipases have included procedures such as fractionation with ethanol, precipitation with ammonium sulfate, gel filtration, ion exchange chromatography, and affinity chromatography. Some of the affinity chromatographic procedures involve palmitoyl cellulose, agarose-linked egg yolk lipoprotein, and dodecylamine agarose (6, 15-18, 20). Kosugi and Suzuki (16, 17) reported using a lipase from Pseudomonas mephitica var. lipolytica to study affinity chromatography.

Affinity chromatography is a type of adsorption chromatography in which the bed material has a biological affinity for the substance to be isolated. The specific adsorptive properties of the bed material are obtained by covalently coupling an appropriate ligand to an insoluble matrix. The binding ligand is able to adsorb from solution the substance to be isolated, desorption being subsequently carried out by changing conditions after unbound substances have been washed away. Affinity chromatography provides opportunities for the isolation of substances according to their biological function, and thus differs

from conventional chromatographic techniques in which separation depends on the physical and chemical properties of the substances to be separated (1).

Complex media have been used as sources of enzyme for the several purification attempts of lipase from bacteria (27). One of the problems encountered in purifying the enzyme is selective concentration of the enzyme from the mixture of substances found in the growth medium. Moreover, the organism typically produces other extracellular products which interfere with purification procedures. In an attempt to avoid some of these problems, the use of washed cell suspension as a source of enzyme was investigated in this study.

To determine lipase activity a rapid and sensitive method for enzyme quantitatation must be employed. One technique for quantitation of microbial lipases is based on the titration of free fatty acids released from the breakdown of glycerides (24). In this procedure the enzyme and substrate mixture are incubated for a prescribed period of time and the fatty acids released are titrated with an alkaline solution. Although the titration yields precise results, it is not easily adapted to testing a large number of samples.

Another assay procedure is based on a biphasic separation of oleic acid from trioleate (4, 8). This technique when combined with the use of specifically-labelled trioleate can be used to accurately quantitate extracellular lipase activity. Isotopically-labelled trioleate increases the sensitivity of the assay since minute quantities of the label can be measured accurately.

The purpose of this study was to purify a lipolytic enzyme from P. aeruginosa. Production of the enzyme by a washed cell suspension combined with the use of affinity chromatography was used for purification procedures. This report also includes some preliminary data on some of the properties of the enzyme.

CHAPTER II

MATERIALS AND METHODS

Organisms

A known lipase-producing strain of Pseudomonas aeruginosa (ATCC 19154) was obtained from the American Type Culture Collection. This strain had been used for the commercial production of lipase (2) and was used as the source of the enzyme for purification and characterization in these studies. The culture was maintained on brain heart infusion agar (Difco). After incubation for 18-24 hours at 37C, the slants were stored at 4C. The strain was sub-cultured at monthly intervals.

Culture Media and Growth Conditions

To obtain the enzyme, the organisms were grown in six 500-ml side-arm flasks. Each of the flasks contained 100 ml of basal medium consisting of 1% (w/v) peptone (Difco) and 1% (v/v) substrate emulsion. The substrate emulsion was composed of one volume of olive oil (Pompeian) and nine volumes of 1% (w/v) polyvinyl alcohol (Matheson Coleman, and Bell) in water. This mixture was treated in a Brinkman Polytron homogenizer (two intervals of 10 sec duration each at a rheostat setting of five) to give an emulsion which remained stable for several hours at room temperature. Outdated blood serum obtained from the blood bank of Wadley Institute of Molecular Medicine was heated at 60C for 30 min to destroy endogenous lipolytic

activity. The heated serum was then added to the emulsion in the ratio of one volume emulsion to nine volumes blood serum. To allow the serum and oil emulsion to stablilize, this mixture was incubated for thirty minutes at 37C before use.

The inoculum consisted of 1.0 ml (1%) of an 18-hr brain heart infusion broth culture which had been diluted with the same broth to a turbidity of 100 Klett units. Turbidity measurements were made using a Klett-Summerson Photoelectric Colorimeter equipped with a green number 54 filter (520-580 nm, with maximum transmission at 540 nm). The cultures were incubated at 37C for 10 hr on an Aquatherm gyrotary shaker at 100 rpm (stroke of one inch; New Brunswick Scientific Co.). The supernatant fraction was obtained by centrifugation of the culture for 15 min in a Beckman J-21C centrifuge at 17,000 x g. The clear liquid was used as the crude enzyme preparation.

In some of the experiments the sedimented cells from 600 ml of culture were pooled, washed three times with 0.9% saline, and resuspended in 20 ml of saline with vortexing to disperse the cells. Three milliliters of the washed cell suspension were inoculated into each of six 250-ml flasks containing 50 ml of deionized water. These flasks were incubated for 90 min at 37C on the gyrotary shaker at 100 rpm.

The cell-free supernatant obtained by centrifugation of the washed cell culture for 15 min at 17,000 x g was lyophilized in a Virtis freeze-dryer. The freeze-dried supernatant was stored in the freezer and resuspended in Tris-HCl buffer (0.02M; pH 8.0) containing 0.2M NaCl and used as needed.

Lipase Assay

Lipase activity was assayed by determining the radiolabelled free fatty acids released during the hydrolysis of the triglyceride substrate emulsion. Glycerol tri[9, 10(n)^{3}H]-oleate was used as the tracer substrate. The labelled trioleate was obtained from Amersham/Searle Corporation (99% radiopurity as determined by thin layer chromatography according to the manufacturer's specification). The "assay substrate" emulsion was prepared similarly to the growth substrate emulsion, except that triolein (Sigma) was used in place of olive oil and 7.5 µl of labelled trioleate per milliliter of substrate were added to the mixture before homogenization.

One hundred microliters of the assay substrate emulsion and 200 µl of NH_4OH/NH_4Cl buffer (0.02M; pH 8.3) were placed in each 20 x 50-mm screw cap tube. The contents of the tubes were equilibrated at 37C in a water bath-shaker (Lab-Line Instruments), and the reaction was started by the addition of 100 µl of enzyme preparation. Incubations were for 2 hr (unless otherwise stated) on a reciprocating shaker at 36 strokes per minute (stroke of 1.5 inches).

The reaction was stopped by the addition of 6.5 ml of a mixture consisting of methanol, chloroform, and n-heptane (1.41:1.25:1; v/v/v). The radioactive free fatty acid was isolated by a modification of the method of Belfrage (4). Two and one-tenth milliliters of potassium carbonate buffer (0.05M; pH 10) were added to the previous mixture, and separation of the phases was accomplished by centrifuging the system at 500 x g for 15 min in a Sorvall GLC-2B centrifuge (Du Pont Company).

A 1.0-ml aliquot of the top, polar, layer containing the free fatty acids was assayed for radioactivity using a liquid scintillation spectrometer (Beckman Model LS-250) with 20 ml of scintillation fluid. The scintillation fluid was prepared from a commercial concentrate (Eastman Concentrate I) with a final composition of 4.0 grams PPO and 0.05 grams POPOP per liter of toluene. The final mixture contained 25% Triton X-100 as a solubilizing agent.

Enzyme activity was calculated according to the formula (28):

$$\underline{\text{Enzyme activity}} = \underline{\text{net cpm}} \times (\underline{1/\text{incubation time}}) \times (\underline{1/\text{sp act}}) \times \underline{3} \times \underline{4.9} \times (\underline{1/0.76})$$

Enzyme activity is expressed as milliunits (mU) in the sample assayed; one mU of enzymatic activity is defined as the release of one nmole of oleic acid per min at 37C, where net cpm = radioactivity in 1.0 ml of upper phase containing the fatty acids, sp act = specific activity of the substrate expressed as cpm per nmole of triglyceride, 3 = the molar ratio of fatty acid released to triglyceride hydrolyzed, 4.9 = the correction factor for the volume of the upper phase, and 1/0.76 = the partitioning coefficient of oleic acid in the extraction system. Incubation time is given in minutes.

Preparation of Crude Lipase Concentrates

All procedures were done at approximately 4C. The culture supernatant fluids from six flask cultures (approximately 550 ml) were obtained by centrifugation as described above and concentrated to approximately 75 ml in an Amicon Hollow Fiber Dialyzer/Concentrator with an exclusion limit of greater than 50,000 molecular

weight. The concentrate was then lyophilized in a freeze-dryer (Virtis Company, Model 10-030). The freeze-dried concentrate was stored in the freezer and resuspended in Tris-HCl buffer (0.02M; pH 8,0) containing 0.2M NaCl as needed.

In some of the experiments, ammonium sulfate was added with gentle stirring, to a final concentration of 75% saturation (9). After 18 hr, the resulting precipitate was recovered by centrifuging the mixture at 17,000 x g. The precipitate was dissolved in a minimum amount (approximately 10 ml) of phosphate buffer (0.05M; pH 8.3) and then dialyzed for 18 hr against fifty volumes of the same buffer with three buffer changes during the period.

Sephadex G-200 Chromatography

A concentrate of the culture supernatant prepared by ammonium sulfate precipitation was fractionated on a gel filtration column. In preparing the column, 20 g of dry Sephadex G-200 (Pharmacia Fine Chemicals, Inc.) were placed in 500 ml of phosphate buffer (0.02M; pH 8.3) and allowed to swell overnight at 4C. The swollen Sephadex was then de-aerated under partial vacuum for 20 min and poured into a 70 x 2.5 cm column. The final height of the gel was 60 cm. The flow rate for the column was approximately 15 ml per hr. The column was allowed to equilibrate for at least 24 hr with the same buffer before a 5 ml sample was placed on the column. Elution was with the same phosphate buffer. Fractions of 5 ml each, were collected by an automatic fraction collector (LKB, Model 2112 Redirac). The protein content of the fractions was estimated by

absorbance at 280 nm in a Gilford UV-Vis Spectrophotometer Model 250. Fractions were also assayed for lipase activity.

Concentrates of the supernatant from the washed cell suspension prepared by lyophilization were also fractionated on Sephadex G-200.

Affinity Chromatography

The column material was prepared by a modification of the procedure of Kosugi and Suzuki (17). In preparing the column 15 g of freeze-dried CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc.) were washed for 15 min with 1mM HCl on a sintered glass filter. The washed material was added to 75 ml of a coupling mixture which consisted of a 1:1 mixture of ethanol and sodium bicarbonate (0.1M) solution containing 0.5M NaCl and 75 mg of n-dodecylamine (Aldrich Chemical Corp.). The mixture was slowly stirred for 2 hr at room temperature on a magnetic stirrer (Sybron/Thermolyne). Unbound dodecyalmine was washed away with coupling mixture and distilled water. The remaining groups on the gel were allowed to react with ethanolamine (1.0M; pH 8.0) for 2 hr. The coupled gel was successively washed three times with the coupling mixture, acetate buffer (0.1M; pH 4.0 containing 1.0M NaCl). The gel then was washed with Tris buffer (0.02M; pH 8,0) containing 0.2M NaCl (equilibration buffer, de-aerated under partial vacuum, and poured into a 30 x 1.5 cm=glass column. The final height of the gel was 15 cm. The flow rate of the column in the downward flow was 25ml/hr. Crude enzyme concentrate, dissolved in the equilibration buffer was allowed to percolate through the column and equilibration buffer was added until no more material

with an absorbance at 280 nm was detected in the eluant. Elution was with the equilibration buffer containing 0.3% (w/v) sodium deoxycholate (Difco). The elution buffer was heated at 100C for 10 min to destroy endogenous lipase in the sodium deoxycholate. Other eluting buffers tested included Tris-HCl buffer containing 0.005M $CaCl_2$, acetone/bicarbonate (1:1) containing 0.5M NaCl, Tris-HCl buffer containing 4% (v/v) glycerol, Tris-HCl buffer containing 30% glycerol, and Tris-HCl buffer containing 1.0M NaCl with and without 20% ethylene glycol. All of the above buffers containined 0.2M NaCl unless otherwise stated.

Fractions of 14 ml each were collected. Protein content of the fractions was estimated by absorbance at 280 nm and fractions were tested for lipase activity as described above.

Ouchterlony Test

Ouchterlony plates were prepared by dissolving 1% agarose (Difco) in 0.85% saline. Sodium azide (Sigma) at a concentration of 0.02% was added as a preservative. Approximately 5 ml was poured into each 60 x 15 mm tissue culture dish (Falcon Plastics). A central 8-mm well was cut in the agar with six wells around it 8 mm from it. A Grafar gel punch assembly (Grafar Corp.) was used to cut the wells.

Anti-human serum albumin was put in the central well. Human serum albumin and enzyme preparation were placed alternately in the outside wells.

CHAPTER III

RESULTS

Properties of Lipase

As previously shown in this laboratory (8), the lipolytic enzyme of Pseudomonas aeruginosa ATCC 19154 is active with a substrate emulsion prepared with olive oil. Figure 1 shows that the enzyme prepared under the same experimental conditions is active with a substrate emulsion prepared with triolein. Production of fatty acids from the purified substrate was linear with time for up to 100 min.

While specificity of the enzyme toward the respective substrates was not determined, the total lipase activity and cell growth in cultures grown with olive oil were the same if triolein was used in the growth medium (Table I).

The effect of pH on enzyme activity was studied using a concentrate prepared by ammonium sulfate precipitation of the culture supernatant as the enzyme source ("Ammonium Sulfate" fraction in Table III). Assay media were prepared with Tris-HCl buffer over the pH range 7.0-8.7. The lipase displayed pH optima at 7.4 and in the range 8.2-8.4 (Figure 2).

Sephadex G-200 Gel Filtration

After lipase activity was concentrated by ammonium sulfate precipitation and passed through a Sephadex G-200 column, a broad

Fig. 1--Lipase activity as a function of incubation time. A cell-free supernatant obtained from a 10 hr culture of P. aeruginosa was used as the enzyme source.

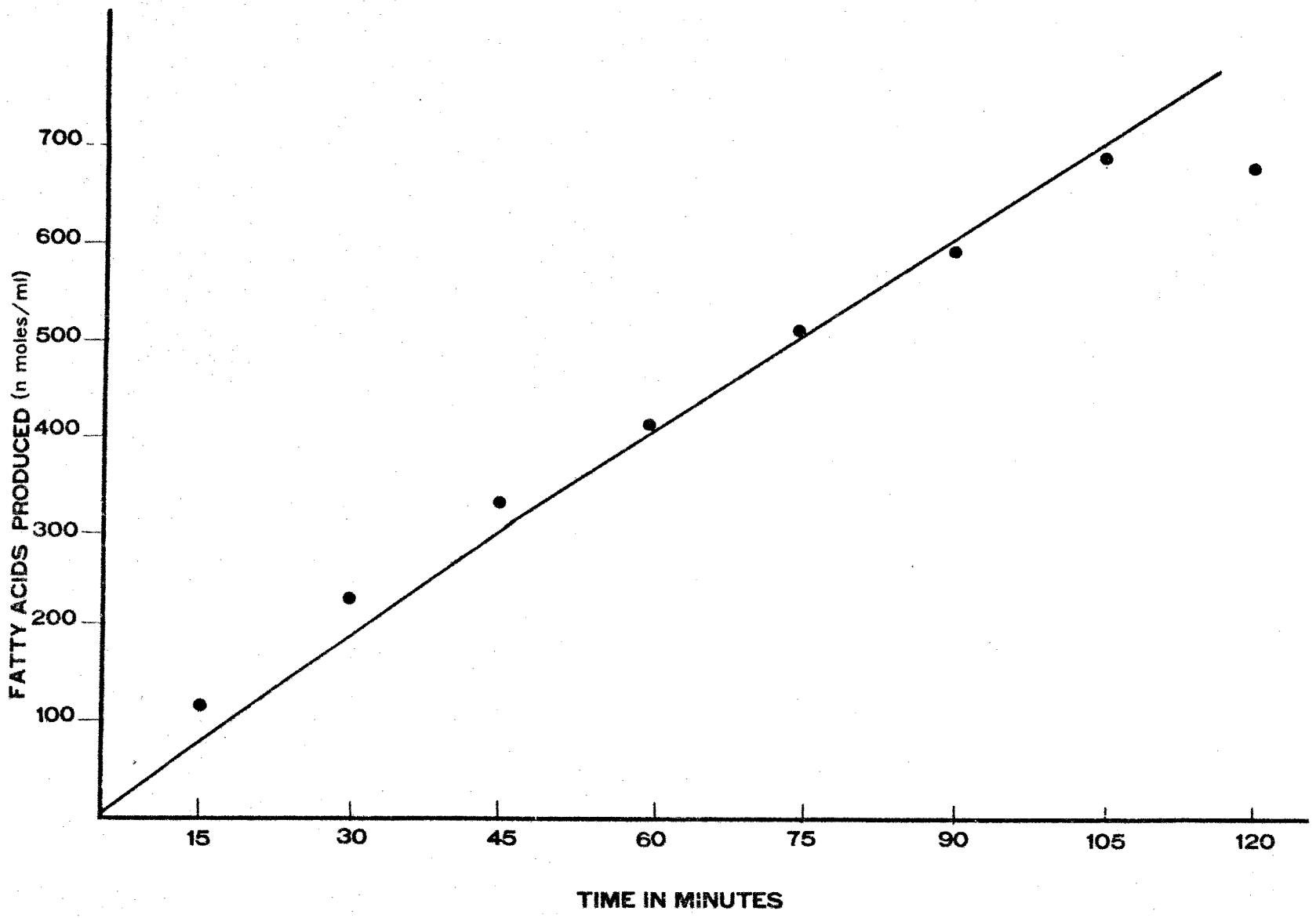

FATTY ACIDS PRODUCED (n moles/ml)
700
600
500
400
300
200
100
15
30
45
60
75
90
105
120
TIME IN MINUTES

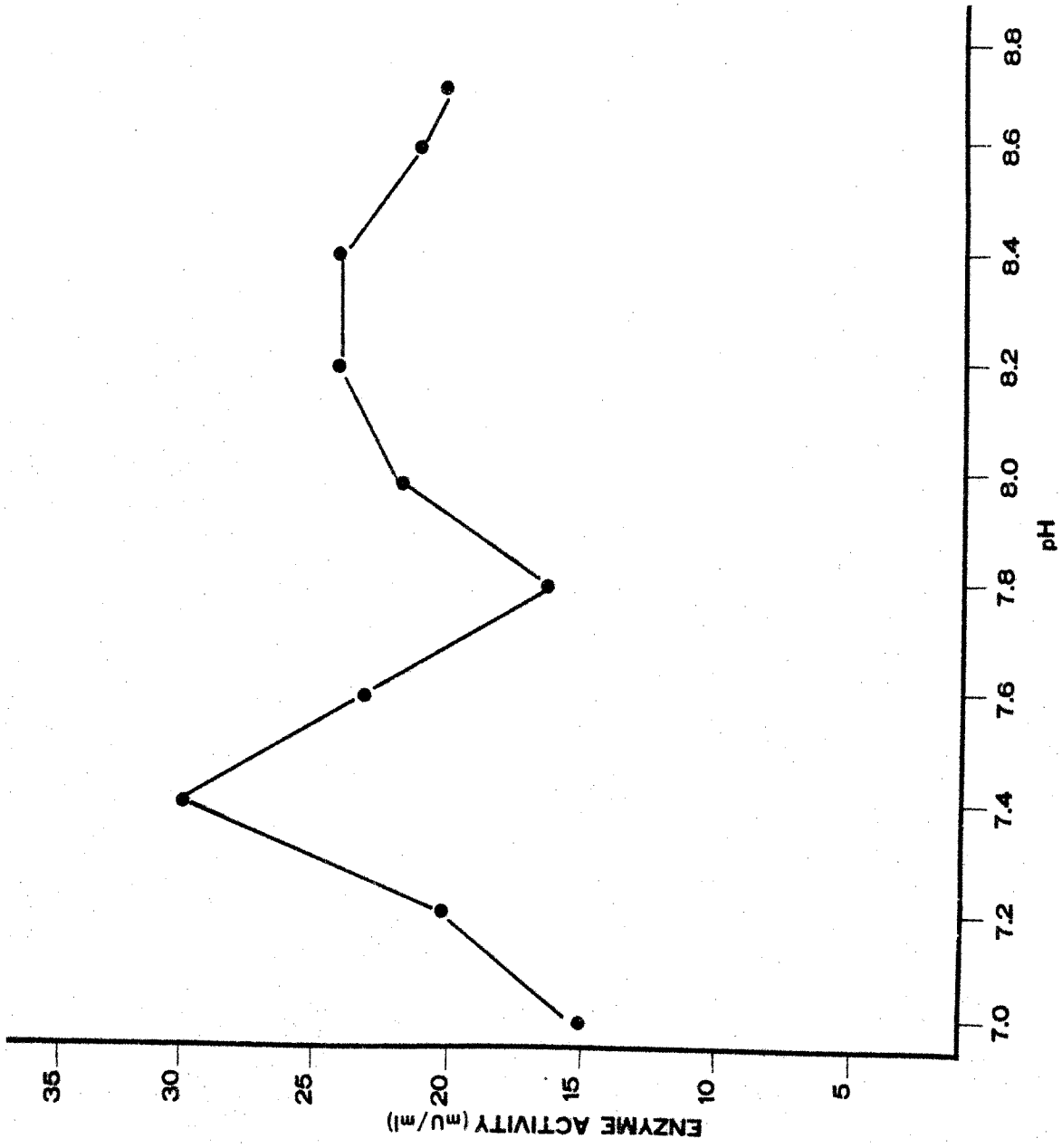
TABLE I

EFFECT OF THE COMPOSITION OF MEDIA ON LIPASE PRODUCTION OF *P. AERUGINOSA*

MEDIUM	GROWTH (Klett units at 540 nm)	ENZYME ACTIVITY (mU/ml of supernatant)
Peptone and olive oil emulsion	140.5	54.5
Peptone and triolein emulsion	138.5	53.3

Note: Bacteria were incubated at 37C for 10 hr with shaking, in 100 ml of media in a 500 ml side-arm flask. Data represent the mean values of two trials.

Fig. 2--Effect of pH on enzyme activity. The fraction obtained by ammonium sulfate precipitation of the supernatant from a 10 hr culture of P. aeruginosa was used as the enzyme source. The reactions were stopped and assayed after a 2 hr incubation period at 37C. All incubations were run in triplicate, with the data points indicating the mean values.


ENZYME ACTIVITY (mU/ml)
35
30
25
20
15
10
5
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
8.6
8.8
pH

peak of activity was obtained (Figure 3). Activity first appeared shortly after the void volume of the elution pattern and was associated with the first two major absorbance peaks. The number of absorbance peaks from the gel filtration column along with the lipase suggested that the applied preparation of enzyme consisted of a complex mixture of substances with a small molecular size differential.

Dodecylamine-Agarose Chromatography

It had been reported (17) that agarose derivatives containing bound aliphatic amines having an even number of carbon chains longer than n-nonylamine had a high affinity for the lipase of Pseudomonas mephitica var. lipolytica.

A column containing n-dodecylamine agarose was prepared by a modification of the method of Kosugi and Suzuki (17). It was found that superior resolution of activity could be obtained by increasing the amount of n-dodecylamine from 0.1 mg/g dry weight Sepharose 4B to 5 mg/g dry weight Sepharose 4B. A solution of the lyophilized powder (1.066 g; "Lyophilized Powder" in Table III) was applied to the column and allowed to percolate through. After the charged column was washed with sufficient buffer to reduce the absorbance at 280 nm to below detectable levels, the buffer was switched to one containing 0.3% (w/v) sodium deoxycholate. As shown in Figure 4, the lipase activity could be eluted with this buffer. The first small peak of enzyme activity shown on the graph is that which came through while the lipase was being adsorbed to the column. The second larger peak of activity is that which was eluted with the sodium deoxycholate. The

Fig. 3--Chromatography of P. aeruginosa lipase on a Sephadex G-200 column. A solution (20 ml) of "Lyophilized Powder" (1.066 g) was applied. The flow rate was approximately 15 ml/hr and 5.0 ml fractions were collected. Other experimental conditions are described in the text.

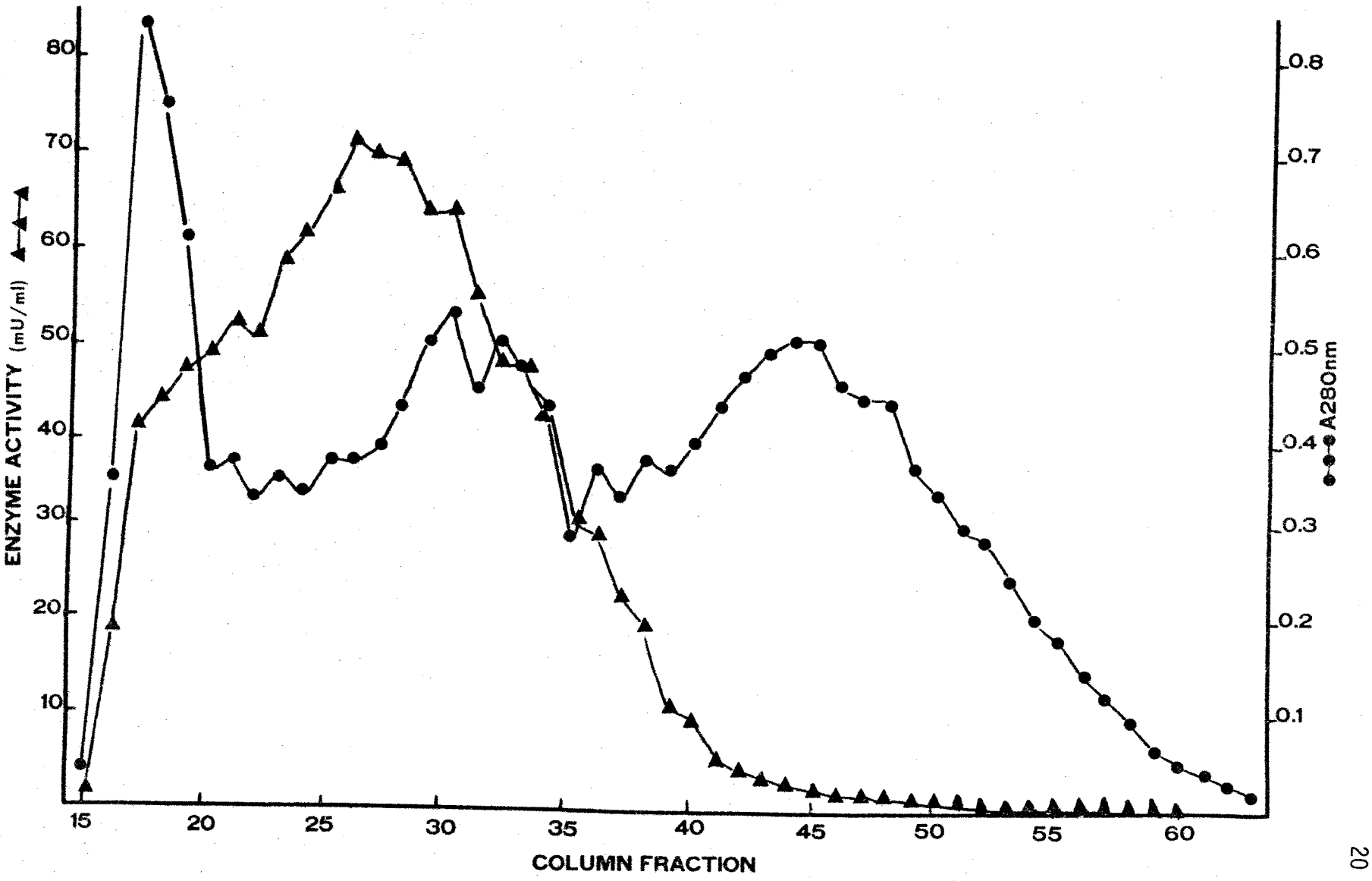
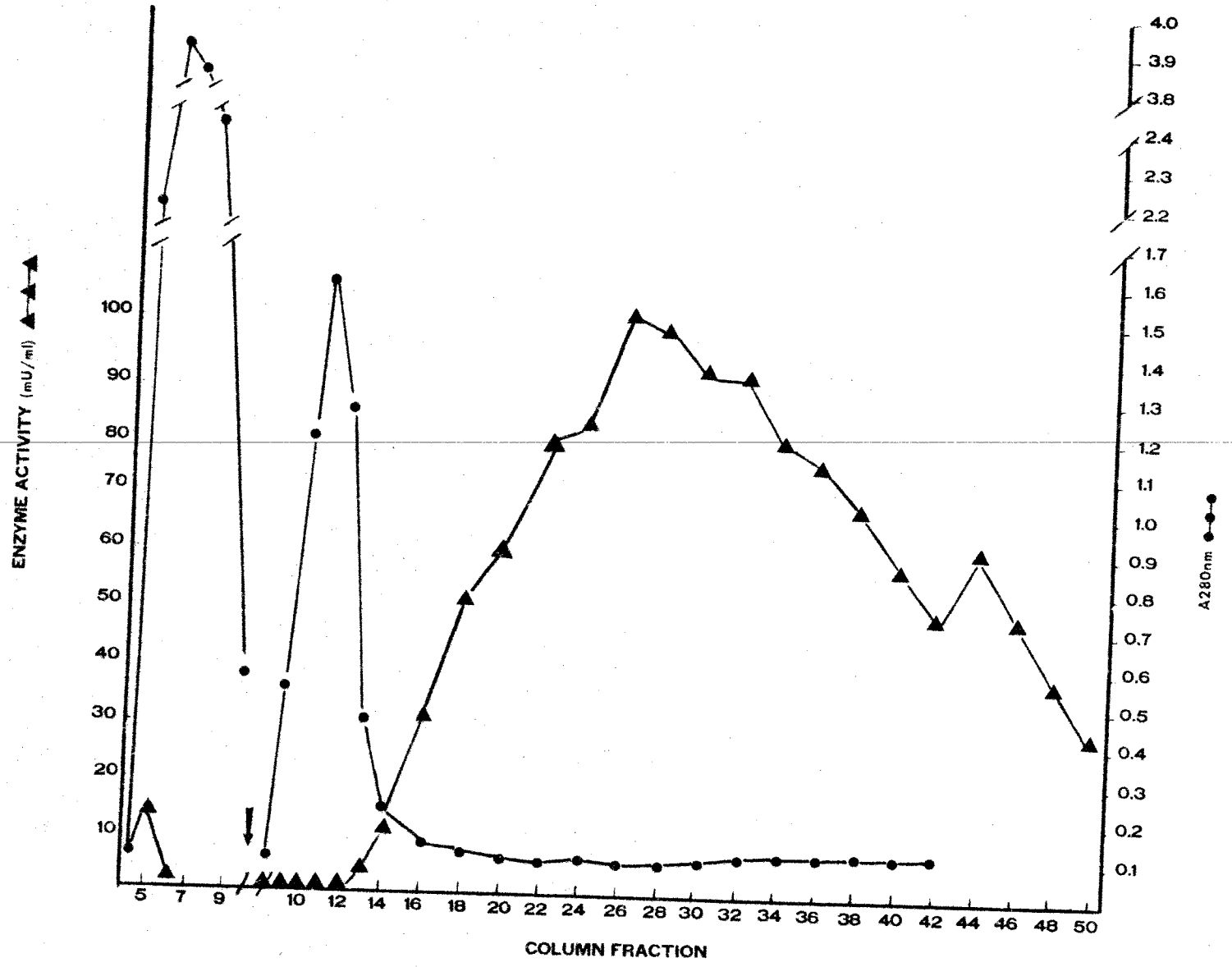

ENZYME ACTIVITY (mU/ml)
80
70
60
50
40
30
20
10
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A280nm
15
20
25
30
35
40
45
50
55
60
COLUMN FRACTION

Fig. 4--Dodecylamine-Sepharose 4B affinity chromatography of P. aeruginosa lipase. The column was eluted first with Tris-Hcl buffer containing 0.2M NaCl and then with Tris-HCl-NaCl buffer containing 0.3% sodium deoxycholate. Buffer change is indicated with an arrow.


ENZYME ACTIVITY (mU/ml)
100
90
80
70
60
50
40
30
20
10
4.0
3.9
3.8
2.4
2.3
2.2
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A280nm
5 7 9 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
COLUMN FRACTION

enzyme activity was separated from the major peak of absorbance. This peak was shown to contain albumin by immunodiffusion plates (Figure 5). The albumin is present in the growth medium and seems to display a similar kind of binding to the n-dodecylamine-agarose column.

In an attempt to determine whether the first peak of activity was due to overloading the column or some other factor, the fraction from the first peak showing the highest activity was reapplied to the column after the column had been thoroughly washed and reequilibrated. Although a portion came through the column during the washing procedure, part (48%) of the total activity recovered was retained and eluted with the sodium deoxycholate (Figure 6).

To determine if n-dodecylamine was responsible for the affinity of the column towards the enzyme, column material was prepared in an identical manner except that the ligand (n-dodecylamine) was not used. When lyophilized supernatant from a 10-hr culture of P. aeruginosa was applied as before, the enzyme came through with the first wash. None was eluted with the sodium deoxycholate (Figure 7).

Effects of Various Compounds on Enzyme Activity and Elution from Dodecylamine-Sepharose 4B Columns

Since sodium deoxycholate was found useful in eluting the enzyme from the column, it was of interest to investigate its effect on the enzyme. A concentrate prepared by ammonium-sulfate precipitation of supernatant of a 10-hr culture of P. aeruginosa was used as the enzyme source and concentrations of sodium deoxycholate ranging from 0 to 1.6% were added. As shown by Figure 8, sodium

Fig. 5--Ouchterlony immunodiffusion in agarose. Center well containg rabbit antiserum (AS) to human serum albumin. Wells labeled AC contain material from pooled fractions (Nos. 10-12) from the dodecylamine-Sepharose 4B column (Fig. 4), concentrated 10-fold, and wells designated SA contain human serum albumin.

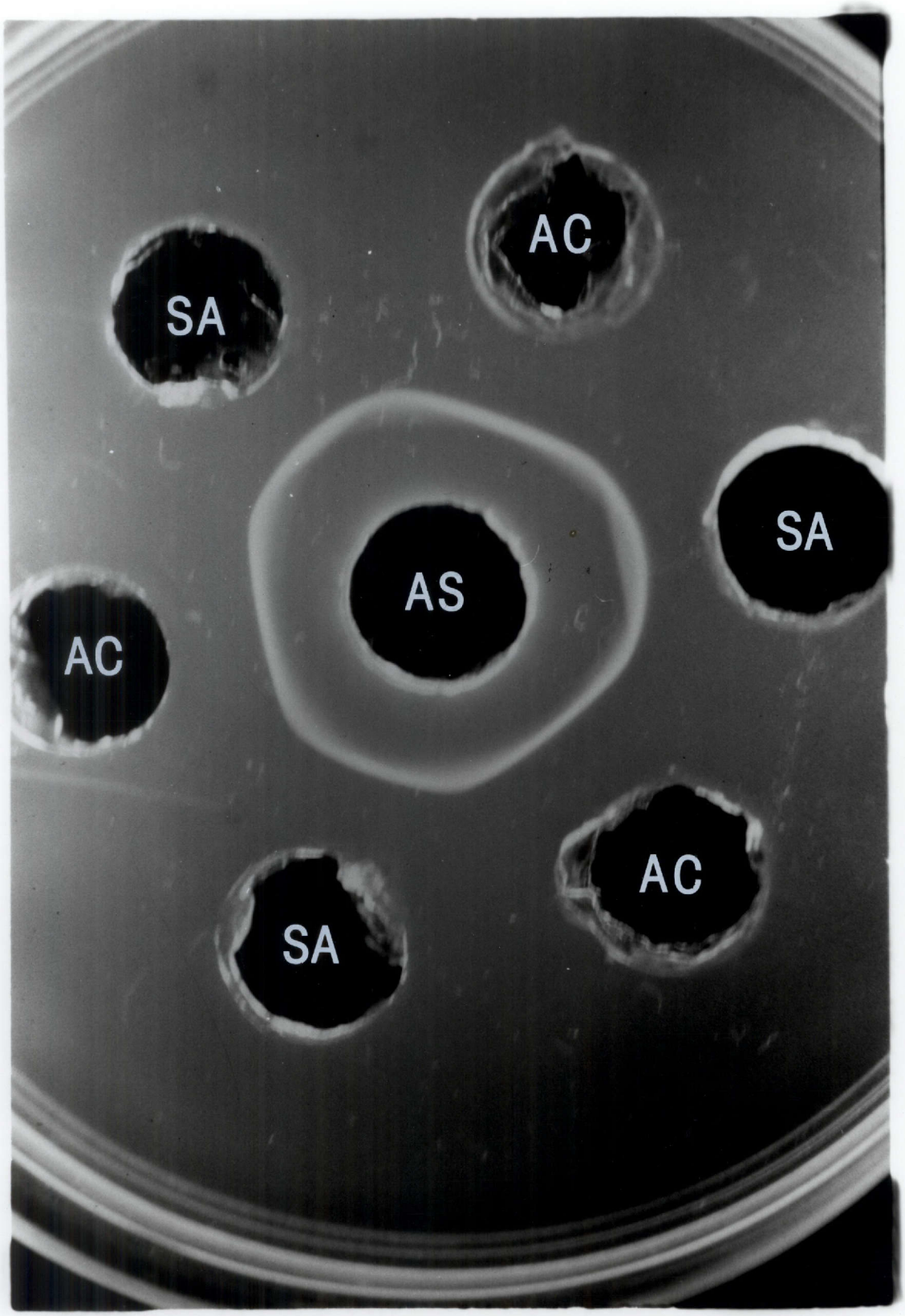

AC
SA
AS
SA
AC
AC
SA

Fig. 6--Rechromatography of lipase on dodecylamine-Sepharose 4B. Fraction No. 6 of the effluent of Fig. 4 was applied to the column. The experimental conditions were otherwise the same as in Fig. 4, with buffer change indicated with the arrow.

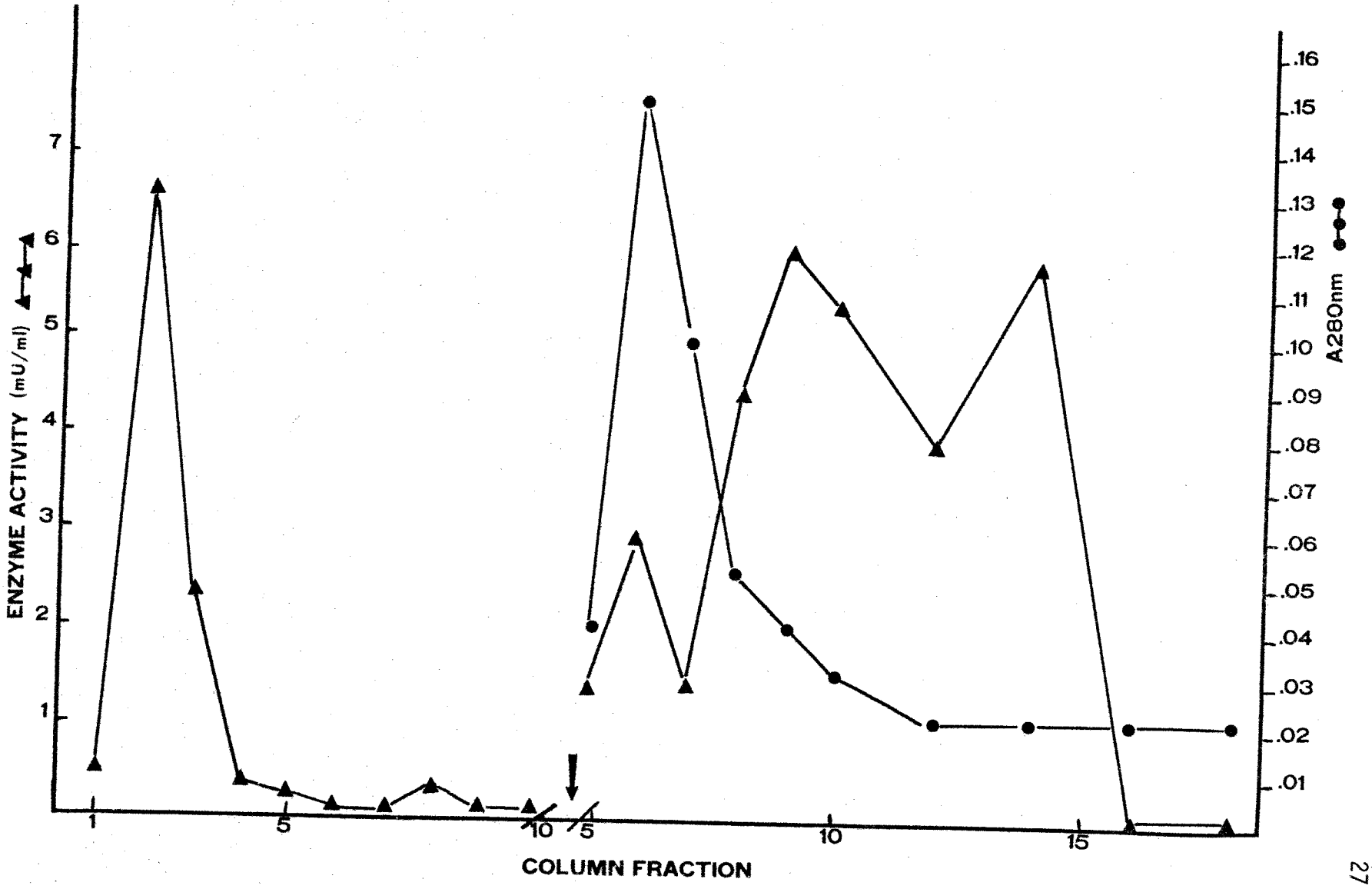

ENZYME ACTIVITY (mU/ml)
A280nm
COLUMN FRACTION
1
5
10
5
10
15
1
2
3
4
5
6
7
.01
.02
.03
.04
.05
.06
.07
.08
.09
.10
.11
.12
.13
.14
.15
.16

Fig. 7--Sepharose 4B chromatography of P. aeruginosa lipase. The experimental conditions were the same as in Fig. 4, except that dodecylamine was not bound to the Sepharose 4B. The enzyme source was the same as in Fig. 4. Buffer change is indicated with an arrow.

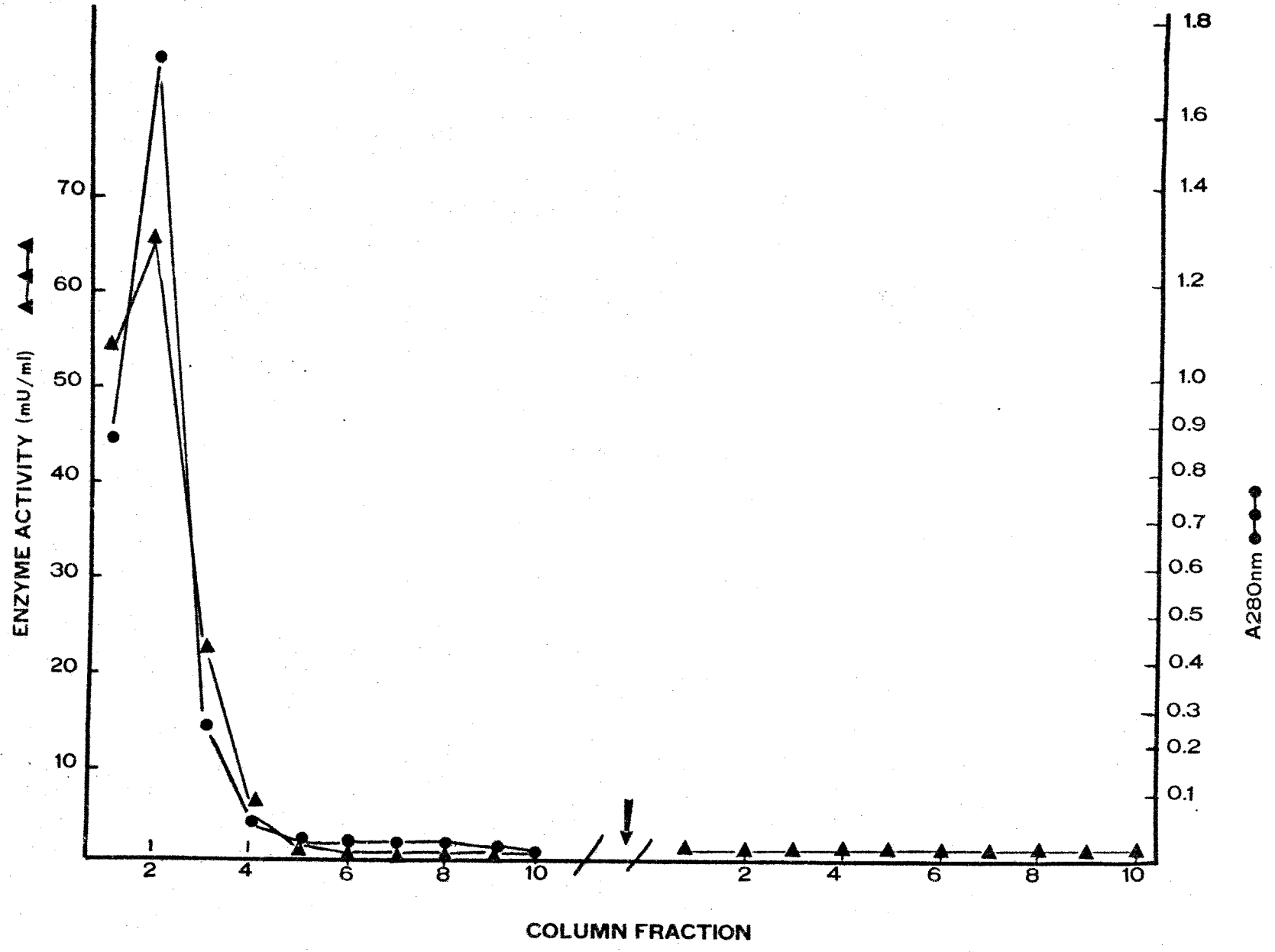
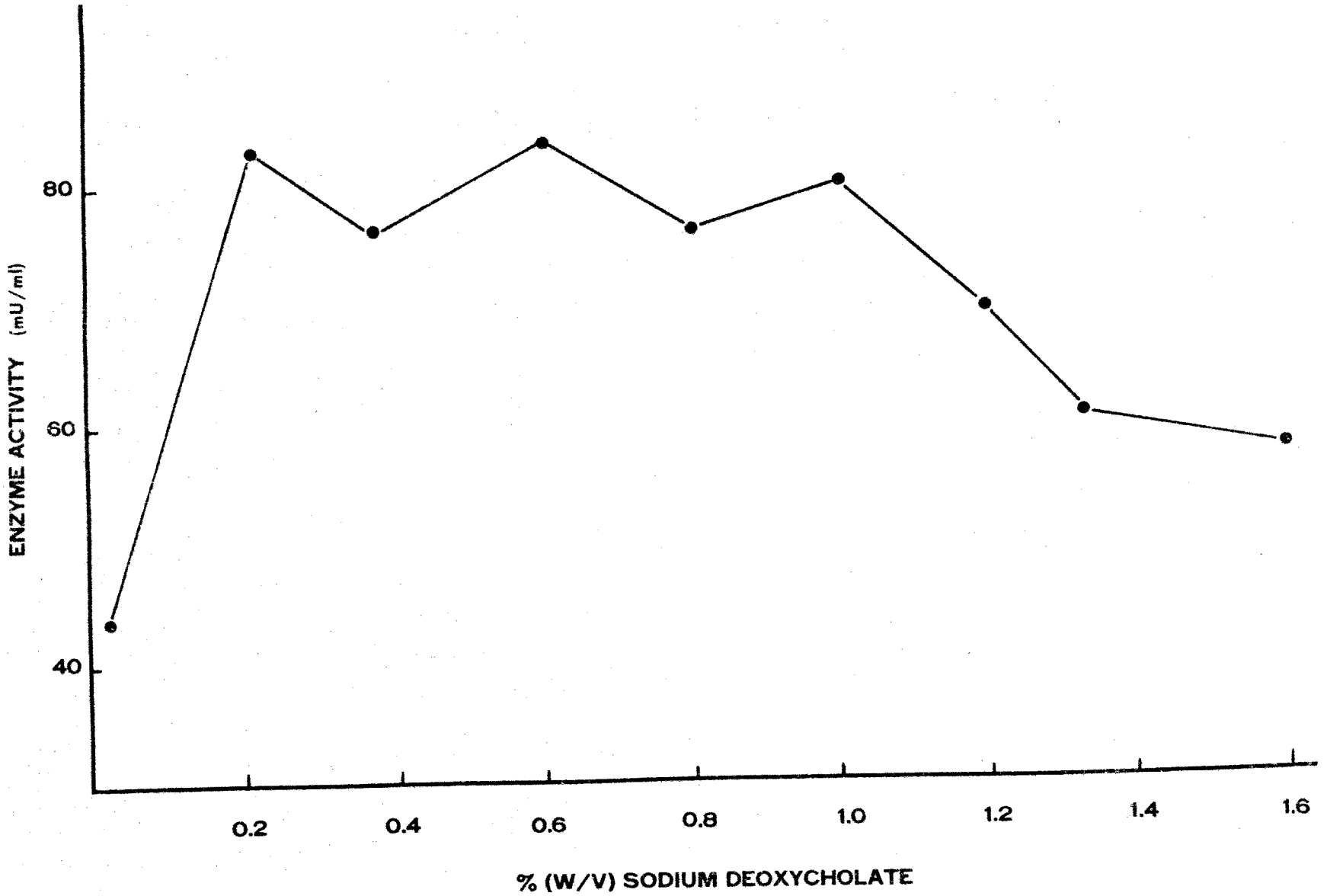

ENZYME ACTIVITY (mU/ml)
70
60
50
40
30
20
10
1.8
1.6
1.4
1.2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A280nm
2
4
6
8
10
2
4
6
8
10
COLUMN FRACTION

Fig. 8--Relation of enzyme activity under standard conditions to sodium deoxycholate concentration. A cell-free concentrated supernatant from a 10 hr culture of P. aeruginosa was used as the enzyme source. The reaction was stopped and assayed after a 2 hr incubation at 37C.


ENZYME ACTIVITY (mU/ml)
80
60
40
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
% (W/V) SODIUM DEOXYCHOLATE

deoxycholate was found to stimulate the enzyme at concentrations up to 0.2%. No further increase in activity was found in the range 0.2% to 1%.

When sodium deoxycholate was used as an eluant in these studies its concentration in the eluted fractions was not known. Therefore, in order to correct for the stimulation of activity due to sodium deoxycholate, the latter was added at a concentration of 0.4% to the buffer used in assaying the fractions; in this manner, the final concentrations of the bile salt would be within the "plateau" region of Figure 8.

In investigating the usefulness of other substances as eluants for the enzyme, compounds were first tested to see what effect they had on enzyme activity. The effect of four of these compounds is shown in Table II. Because both Triton X-100 and Tween 80 completely inhibited the enzyme activity at the concentrations tested, neither was used as an eluant. Although glycerol and ethylene glycol had little or no effect on the enzyme at the concentrations tested, neither of the compounds eluted the enzyme from the column as shown in Figure 9. Neither acetone/bicarbonate buffer containing 0.5M NaCl nor Tris-HCl buffer containing 5mM $CaCl_2$ would elute the enzyme from the column.

Production of Lipase in Washed-Cell Suspensions

Results to this point suggested that blood plasma and other materials in the complex growth medium were causing difficulties in purifying the enzyme. An experiment was undertaken to investigate

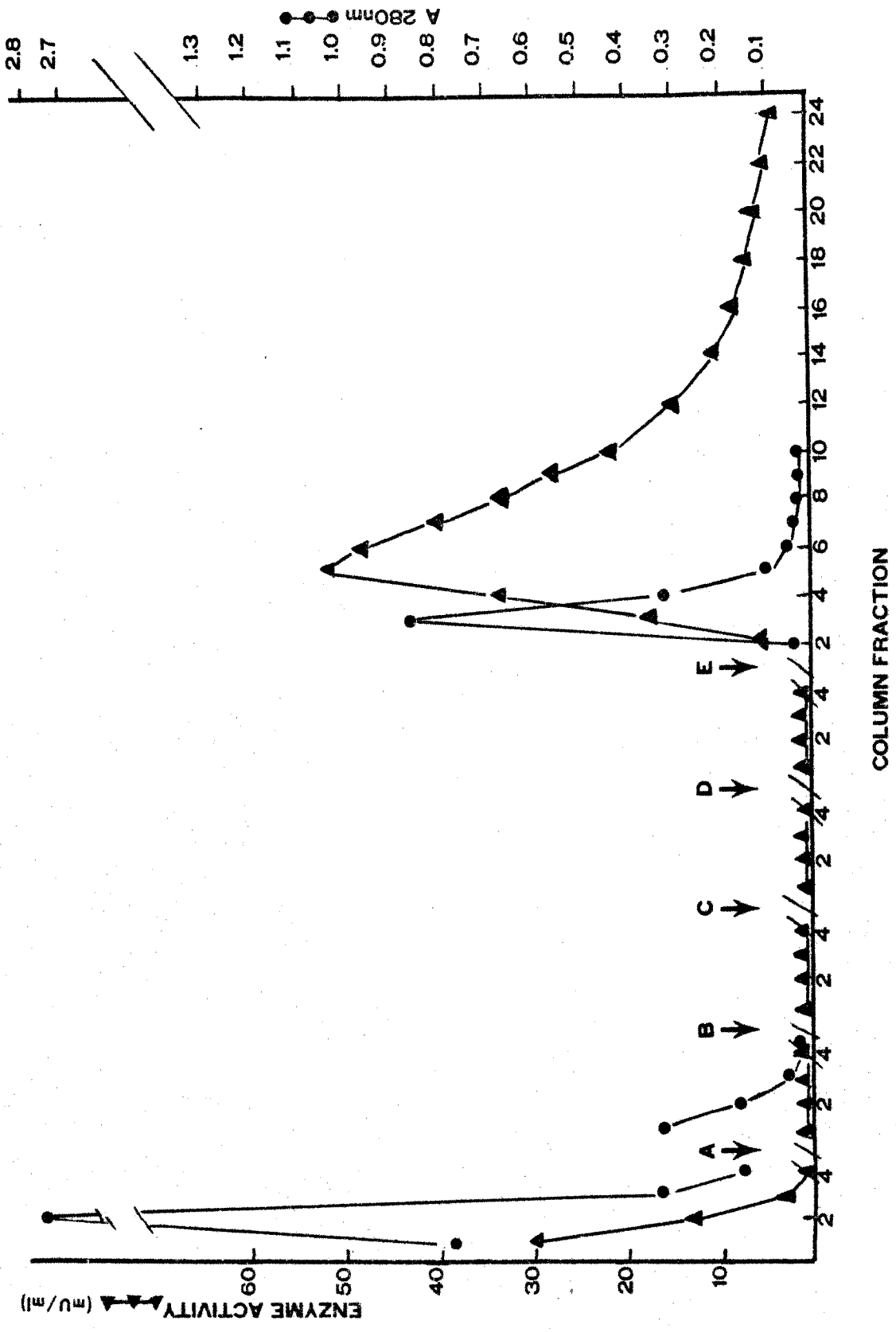
TABLE II

EFFECTS OF COMPOUNDS ON THE ENZYME

COMPOUND	FINAL CONCENTRATION (%, w/v)	RELATIVE ACTIVITY (%)
Triton X-100	0.2	0
Tween 80	0.2	0
Glycerol	0.4	100
Ethylene glycol	20.0	110

The enzyme activity was measured by the standard assay method with the compound at the indicated final concentration. The activity with the addition of each compound is expressed in percent relative to that in the absence of the compound.

Fig. 9--Elution pattern of P. aeruginosa lipase from dodecylamine-Sepharose 4B. A sample (10 ml) containing lipase (1080 milliunits) was applied to the column as described in the Materials and Methods section. Elution was attempted by varying the conditions as indicated by the arrows. A designates the start of elution with Tris-HCl buffer containing 1.0M NaCl; B, that with Tris-HCl buffer containing 4.0% (v/v) glycerol; C, that with Tris-HCl buffer containing 30% (v/v) glycerol; D, that with Tris-HCl buffer containing 20% (v/v) ethylene glycol; E, that with Tris-HCl buffer containing 0.3% (w/v) sodium deoxycholate.


ENZYME ACTIVITY (mU/ml)
A 280nm
COLUMN FRACTION
A
B
C
D
E

a new method for obtaining the enzyme produced by cells in the absence of blood plasma. The cells were grown for 10 hr in the standard media. At the end of 10 hr the cells were washed and resuspended to the original cell density by dispensing into flasks containing either deionized water, water plus substrate (olive oil emulsion and blood plasma), or 1% peptone plus substrate. The cell suspensions were incubated with shaking at 37C and samples were removed at intervals as indicated by the data points in Figure 10. The cells were removed by centrifugation and the supernatant portions were assayed for enzyme activity. Almost no enzyme activity was detected in the flask containing cells resuspended in peptone and substrate. However, levels of enzyme in the supernatant portions were found to increase linearly for a 90 min period when cells were resuspended in water or in water plus substrate emulsion.

The supernatants from these suspensions which had been incubated for a 90-min period were lyophilized. It was found that little or no activity of the dried powders was lost over a 2 month period.

Gel Filtration of Lipase Produced in Cell Suspension through Sephadex G-200

The lyophilized powder prepared from the supernatant portion of the water/cell suspension was dissolved in 4.0 ml buffer and 3.0 ml applied to a Sephadex G-200 column. The results are shown in Figure 11. One peak of protein was observed. All the enzyme activity was associated with this peak.

Fig. 10--Lipase production by washed-cell suspensions of P. aeruginosa over a 3 hr period. (■-■-■) Flasks contained 1.0% peptone and 1.0% substrate emulsion; (●-●-●) Flasks contained water and 1.0% substrate emulsion; (▲-▲-▲) Flasks contained deionized water.

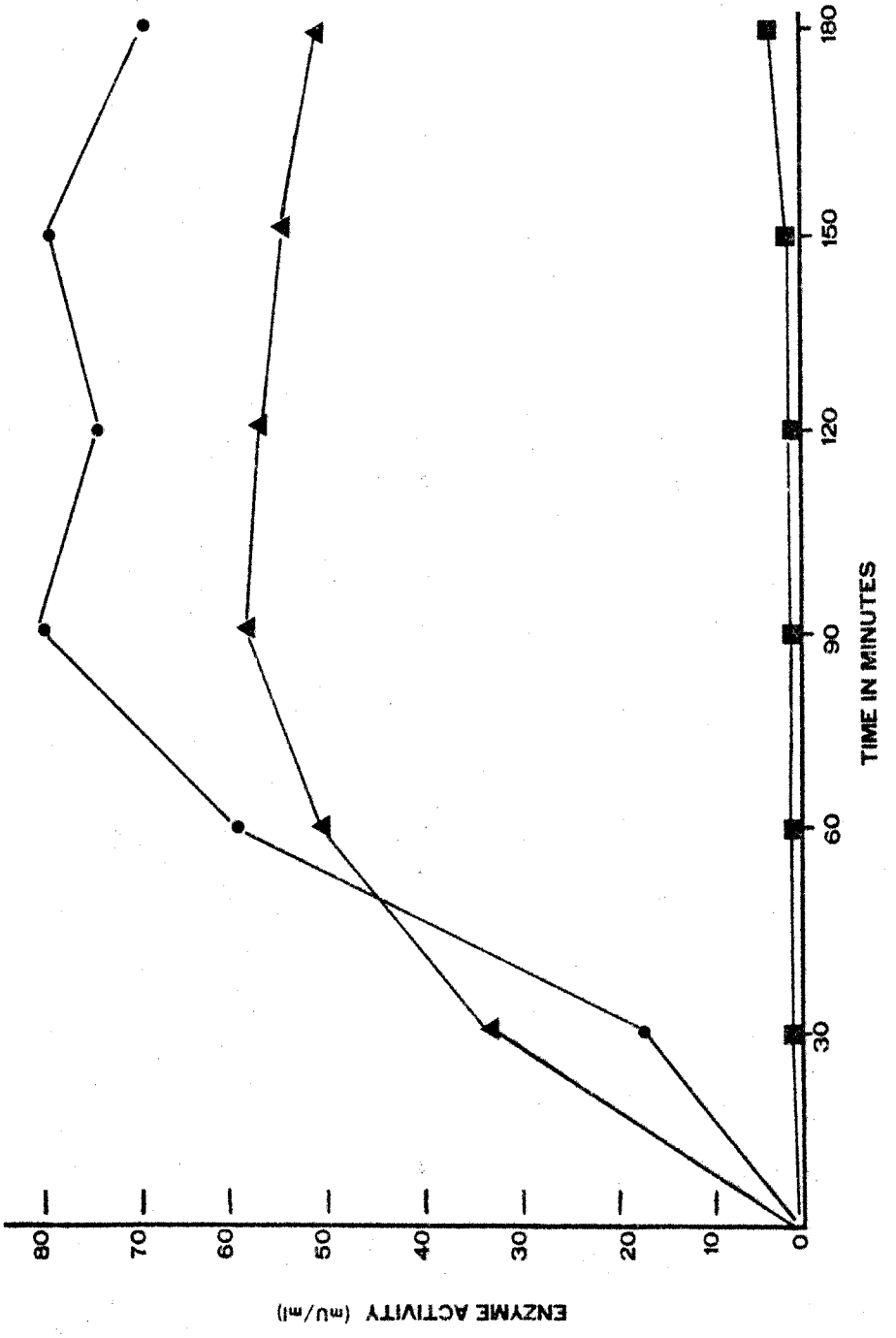
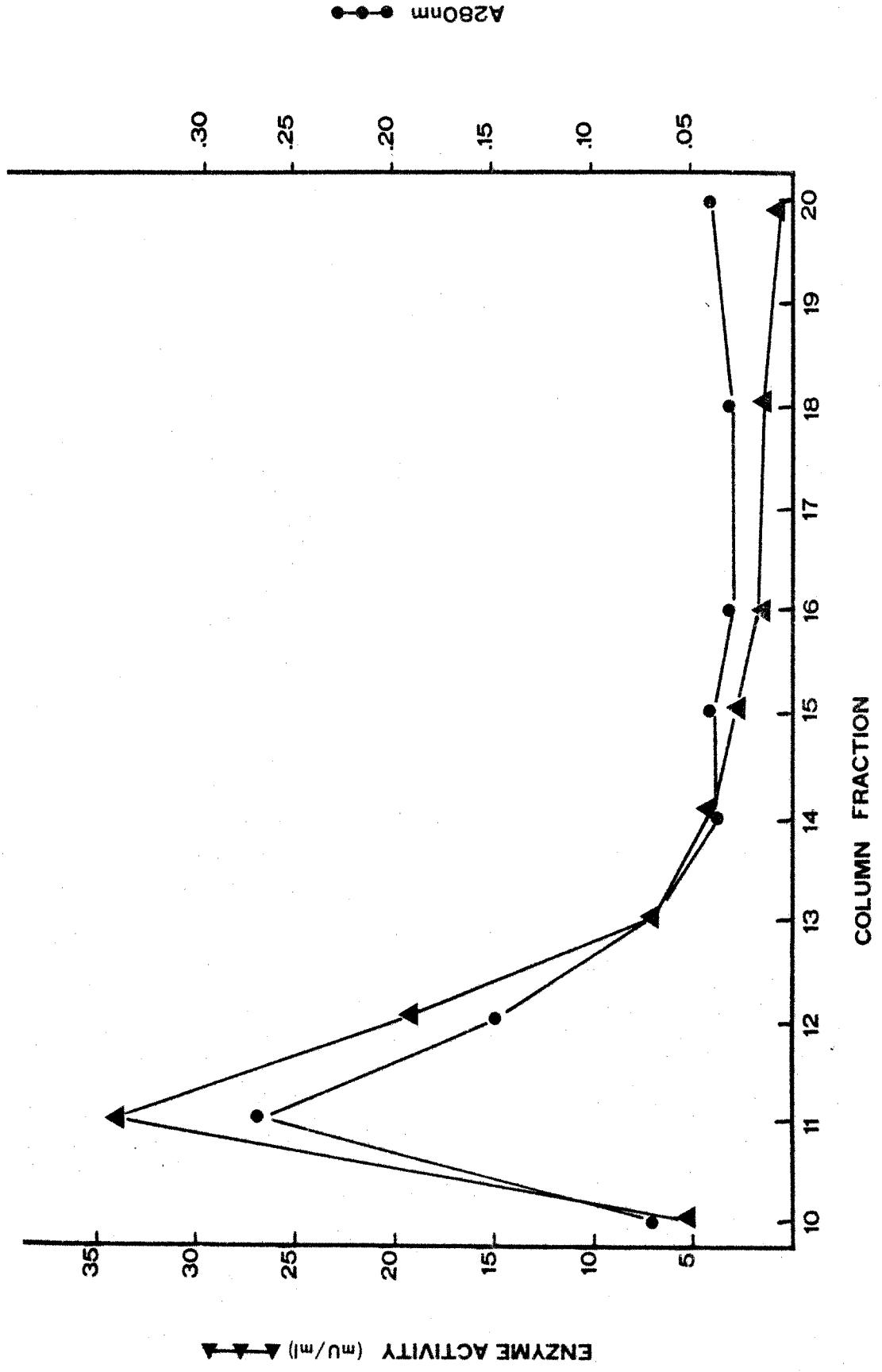

ENZYME ACTIVITY (mU/ml)
0
10
20
30
40
50
60
70
80
30
60
90
120
150
180
TIME IN MINUTES

Fig. 11--Elution pattern from Sephadex G-200 using lyophilized supernatant from washed cells resuspended in water. A 3 ml sample was applied to the column. Fractions of 5 ml were collected at a flow rate of 15 ml per hr.


A280nm
.30
.25
.20
.15
.10
.05
ENZYME ACTIVITY (mU/ml)
35
30
25
20
15
10
5
10
11
12
13
14
15
16
17
18
19
20
COLUMN FRACTION

The lyophilized supernatant from the washed cell suspension in water plus substrate emulsion was also fractionated on a Sephadex G-200 column, and the results are shown in Figure 12. One major peak of protein was observed. A large peak of enzyme activity which was associated with this peak together with a smaller peak of activity which trailed for several fractions were observed. Almost no absorbance at 280 nm was associated with the latter enzyme activity.

Dodecylamine-Agarose Chromatography

A solution of the lyophilized powder prepared from the supernatant portion of the washed cell suspension (0.118 g) was applied to the n-dodecylamine-agarose column and allowed to percolate through. As shown by Figure 13 a small peak of enzyme activity came through while the lipase was being adsorbed to the column. The second large peak of activity is that which was eluted with the sodium deoxycholate. The enzyme activity is separated from the major peak of protein.

Table III shows a summary of the purification procedures for the P. aeruginosa lipase. The purification from the affinity column was from 65- to 95-fold. The recovery of this step in the procedure was 5.8%.

Fig. 12--Elution pattern of P. aeruginosa lyophilized supernatant from washed cells resuspended in water containing 1.0% substrate (olive oil emulsion and blood plasma). Fractions of 5 ml were collected at a flow rate of 15 ml per hr.

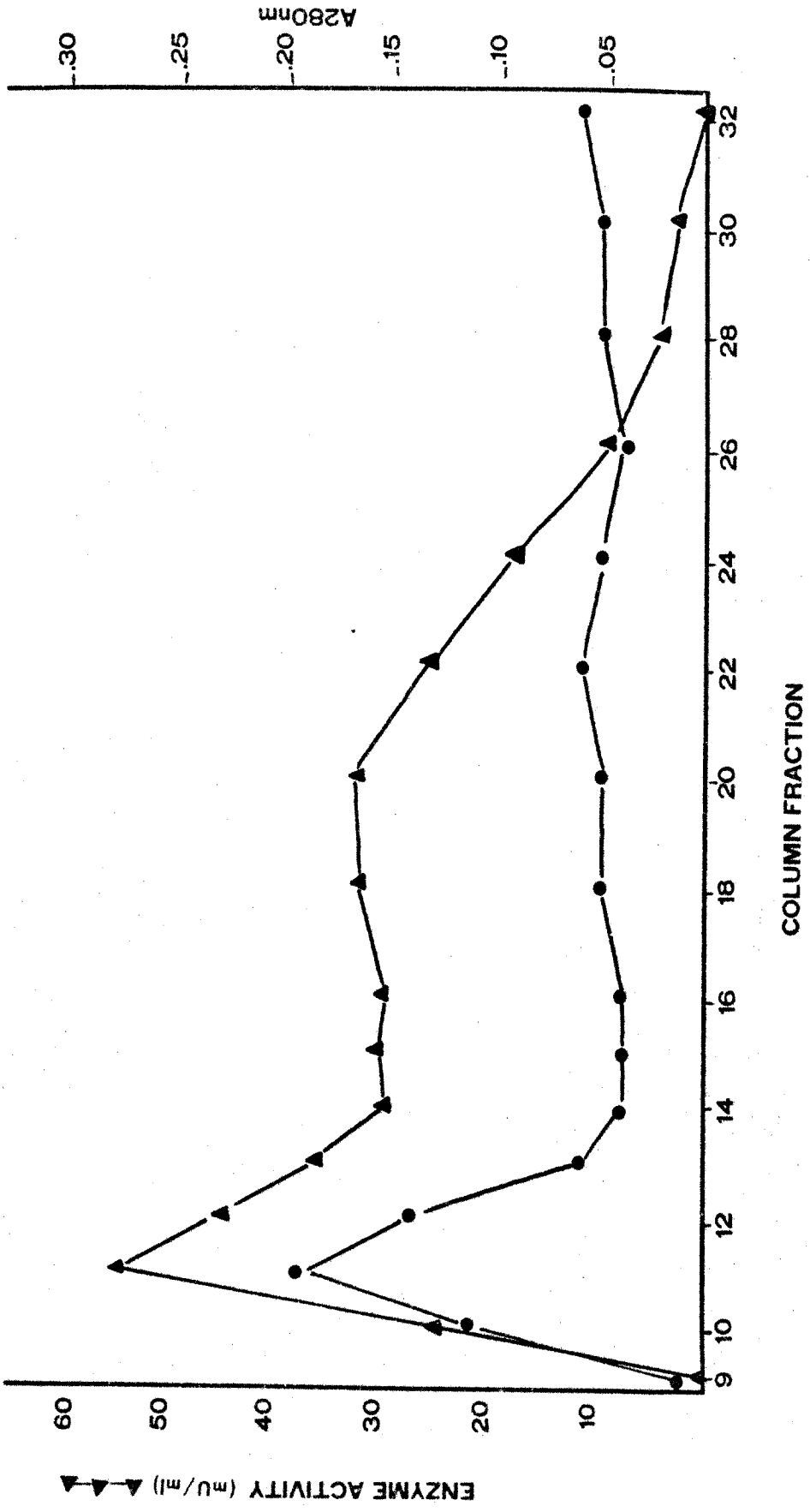

A280nm
.30
.25
.20
.15
.10
.05
ENZYME ACTIVITY (mu/ml)
60
50
40
30
20
10
9
10
12
14
16
18
20
22
24
26
28
30
32
COLUMN FRACTION

Fig. 13--Binding and elution of lyophilized supernatant from washed cells resuspended in water on a dodecylamine-Sepharose 4B affinity column. The column was eluted first with Tris-HCl buffer containing 0.2M NaCl and then with Tris-HCl buffer containing 0.3% sodium deoxycholate. Buffer change is indicated with an arrow.

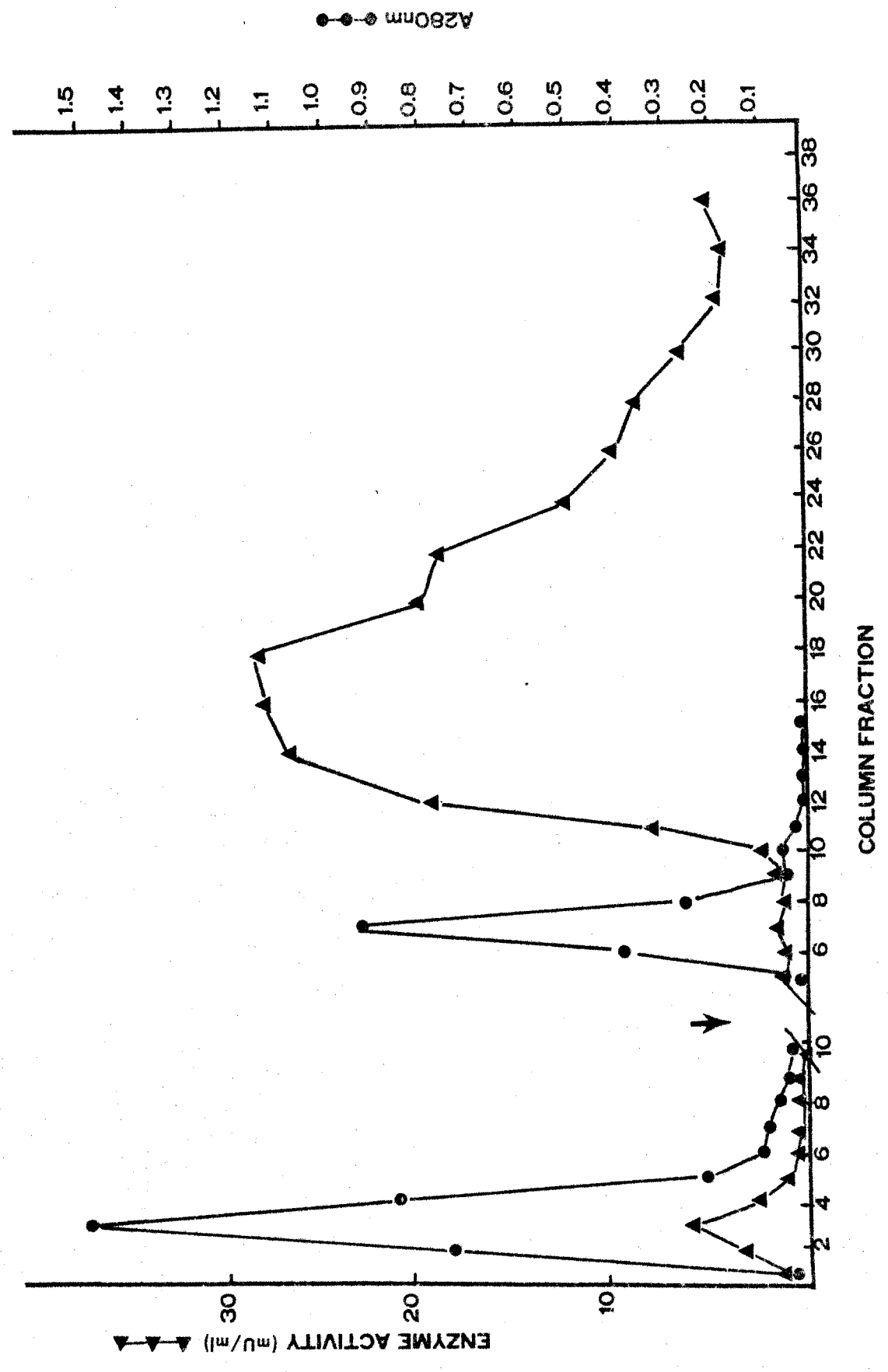

A280nm
ENZYME ACTIVITY (mU/ml)
COLUMN FRACTION

TABLE III

SUMMARY OF PURIFICATION OF P. AERUGINOSA LIPASE

Step in Purification	Volume (ml)	Protein[g] (mg/ml)	Total Protein (mg)
Crude culture supernatant[a]	584.5	3.9	2280
Concentrate prepared by ultrafiltration[b]	93.0	6.2	577
$(NH_4)_2SO_4$ Precipitate[c]	10.5	22.5	235
Lyophilized Powder (Resuspended)[d]	6.0	4.0	24
Fractions from affinity[e]			
26	1.5	0.08	0.12
28	1.5	0.08	0.12
30	1.5	0.09	0.13
32	1.5	0.1	0.15
Washed cell suspension supernatant[f]	275.0	0.5	139

[a]Culture supernatant at 10 hrs obtained centrifugation at 17,000 x g for 15 min.

[b]Culture supernatant (above) concentrated in an Amicon Hollow Fiber Concentrator as described in the text

[c]Concentrated supernatant prepared by ammonium sulfate precipitation as described in text

[d]Concentrated culture supernatant prepared by lyophilization as described in text

[e]Fractions from affinity column (Fig. 4)

[f]Washed cell suspension prepared as described in text

TABLE III--Continued

Enzyme Activity (mU/ml)	Specific Activity[h] (mU/mg)	Total Activity (mU)	Fold Purification	% Recovery[k]
53	14	30970		100
82	13	7626	0.9	24.6
113	5	1187	0.4	3.8
166	42	996	3.0	3.2
102	1275	153	91.0	0.5
99	1242	149	88.7	0.5
91	1054	137	75.3	0.4
90	900	135	64.3	0.4
33	65	9075	4.7	29.2

[g]Protein estimated by absorbance at 280 nm as described in text

[h]Specific activity = munits/mg protein

[i]Total activity = specific activity x total amount of protein = total number of enzyme units

[j]Fold purification = $\frac{\text{specific activity (step n)}}{\text{specific activity (reference)}}$

[k]%Recovery = $\frac{\text{total activity (step n)}}{\text{total activity (reference)}}$

CHAPTER IV

DISCUSSION

The bilayer-radiolabelled lipase assay system was shown to be applicable for use with the lipase from Pseudomonas aeruginosa. Either olive oil or triolein could be used in the assay substrate mixture; however, when triolein was used, the assay could be quantitated and the activity expressed in units thus making it easier to compare results from different experiments.

It was found that the lipase in the concentrate prepared by ammonium sulfate precipitation displayed two pH optima. The fact that a crude preparation was used might suggest that a contaminant interacting with the enzyme may have accounted for the two pH optima or that more than one enzyme was present. Horiuti and Imamura (15) recently reported finding at least two lipase isoenzymes in culture supernatant fluids of Chromobacterium viscosum. The isoenzymes displayed pH optima of around 6 and 8, respectively; each was in a homogeneous state on sodium dodecylsulfate-polyacrylamide gel electrophoresis. An alternative explanation for the results reported here is that the enzyme may have two pH optima; however, Mourey (26) suggested that this is an uncommon event in bacteria.

Chromatography of the enzyme concentrate prepared by ammonium sulfate precipitation on Sephadex G-200 suggested that there were many different proteins of similar molecular size differential in the

preparation applied. The lipase came through shortly after the void volume and without complete resolution, in a pattern similar to that described by Downey and Andrews (10) for mammalian lipases. They suggested, on the basis of their results, that the column fractions could either contain a high-molecular-weight form of the enzyme, a highly aggregated form of the enzyme, or, a smaller enzyme molecule associated with larger (inactive) proteins.

Since the separation on Sephadex G-200 was not successful at this point, the technique of affinity chromatography was attempted as a purification procedure. Dodecylamine, selected as the ligand, was attached to an agarose matrix (Sepharose 4B). Many components of the crude mixture which was applied to the column passed directly through the column. The enzyme could be eluted from the column by changing the buffer conditions. This was done by adding sodium deoxycholate to the buffer.

The ligand was shown to interact with at least one other protein in the crude mixture. This protein was human albumin, a component of the substrate emulsion added to the growth medium. Effective separation of the lipase and albumin was not obtained with the different buffer conditions tested, suggesting that albumin may bind to the n-dodecyalmine agarose in a manner similar to that of the enzyme.

That n-dodecylamine was responsible for the adsorption of lipase to the column was shown by the column containing Sepharose alone; the lipase passed through the column directly, and none of the enzyme was eluted with buffer containing sodium deoxycholate.

A portion of the enzyme invariably passed through the column in the wash, a factor which lowered the maximal recovery of the enzyme using this technique. Incomplete removal of lipase from the solution could have been due to the presence of more than one enzyme in the crude protein preparation, with the n-dodecylamine-agarose having specificity for only certain type(s) of lipase. It is suggested that several enzymes could successively degrade the triolein to smaller and smaller units. One of the enzymes could be responsible for hydrolyzing triolein to diolein or monolein; a second could change the resulting glyceride to the constituent glycerol and fatty acid. One could assume that the optimal conditions for pH for the individual enzymes in this system differ; therefore, the observation of two pH optima (see above) is consistent with this explanation. The use of various substrates might be of considerable help in the determination of precise number of enzymes involved in completely hydrolyzing the triolein molecule.

An alternative explanation for the appearance of lipase in the wash is that the n-dodecylamine derivative may have been quickly saturated with enzyme (column overloading). In order to explore this possibility, material which had passed through the column directly was reapplied to the column, which had first been thoroughly washed and reequilibrated. Because approximately one-half of the recovered activity was retained and subsequently eluted with sodium deoxycholate, it was concluded that column overloading was at least partly responsible for the observation.

It had been shown that some affinity chromatographic procedures, originally thought to depend on biologically specific adsorption, depend largely on generalized adsoprtion effects related to gross physico-chemical properties rather than active-site specificities (3). Binding may be through specific affinity, hydrophobic interactions, hydrophyllic interactions, or electrostatic forces. Binding can also result from electrostatic forces in cooperation with hydrophobic forces (13, 16, 31, 34). Hofstee (14) showed that ethylene glycol decreases the polarity of the medium and tends to weaken hydrophobic bonding. He also showed that the reversal of binding by the addition of salt indicates that hydrophobic bonding is aided by electrostatic forces.

Neither 1.0M NaCl nor twenty percent ethylene glycol desorbed the enzyme from the column which suggested that binding of the enzyme to the ligand, n-dodecylamine, was not primarily by way of hydrophobic or electrostatic forces. Determining whether or not specific affinity is responsible for adsorbing the enzyme to n-dodecyalmine will require further study.

Since P. aeruginosa may be part of the normal intestinal flora (35) and physiological concentrations of deoxycholate (0.2-0.4%) were shown to stimulate lipase activity, there may be a physiological relation between deoxycholate and lipase in the gut. Bile salts, which include deoxycholate, are emptied into the digestive tract during the digestive periods where they act as anionic detergents (12, 19, 33). Because the bile detergents, which function to emulsify and stabilize lipids,

accelerate the reactions catalyzed by lipolytic enzymes elaborated by the pancreas, it seems reasonable to expect that they might also accelerate the reactions of the pseudomonal lipase.

Deoxycholate has been shown to interact with fatty acids forming complexes which are called choleic acids (11). If fatty acids inhibited the activity of the lipase, effective removal of the acids, which are the end products of the breakdown of triglycerides, by deoxycholate may also explain the apparent stimulation of activity when deoxycholate is present.

The complex medium used for enzyme production by P. aeruginosa was shown to contain materials which were partly responsible for the difficulties in purifying the enzyme. Because cells in the washed suspension produced substantial amounts of enzyme in water, numbers of extraneous materials could be reduced and a cleaner preparation was obtained. For these reasons, this method was used to obtain the enzyme. Production of a lipase by a washed cell suspension, which previously had not been reported, thus appears to represent a method which may be simpler and more useful in purification procedures than other methods.

When a solution of the lyophilized powder of the supernatant from the washed cell suspension was applied to a n-dodecylamine-Sepharose 4B column, activity eluted in a pattern similar to that obtained using the preparation from the supernatant of the growth culture. It was noted that the enzyme eluted following a protein peak. That trace quantities of albumin carried over from the growth medium may

have accounted for this peak was suggested by its appearance; however, albumin was not detected by immunodiffusion tests.

The activity of the washed cell suspension was found to be 4.5 fold higher than the activity of the 10 hr culture supernatant; because of this property, the ease of manipulation, and its suitability as starting material for affinity chromatography, the washed cell suspension offers an attractive alternate enzyme source. These procedures may prove useful in solving problems which occur in studies on bacterial lipase.

BIBLIOGRAPHY

1. Affinity Chromatography. Principles and Methods. 1973. Rahms i Lund, Sweden.

2. American Type Culture Collection, 1974. Catalogue of strains, 11th ed., p. 89.

3. Barry, Standish and Padraig O'Carra. 1973. Affinity chromatography of nicotinamide-adenine dinucleotide-linked dehydrogenases on immobilized derivatives of the dinucleotide. Biochemistry Journal. 135:595-607.

4. Belfrage, Per and Martha Vaughan. 1969. Simple liquid-liquid partition system for isolation of labeled oleic acid from mixtures with glycerides. Journal of Lipid Research. 10:341-344.

5. Bijsterveld, O. P. Van. 1976. Lipolytic Activity of Staphylococcus aureus from different sources. Journal of Medical Microbiology. 9:225-228.

6. Breuil, Colette and D. J. Kushner. 1975. Partial purification and characterization of the lipase of a facultatively psychrophilic bacterium (Acinetobacter O_{16}). Canadian Journal of Microbioloby. 21:434-441.

7. Carney, Shriley A., R. E. Dyster, and R. J. Jones. 1973. The invasion of burned skin by Pseudomonas aeruginosa. British Journal of Dermatology. 88:539-545.

8. Christensen, John N. "An assay method for determining extracellular lipases from Pseudomonas aeruginosa," master's thesis, Department of Biological Sciences, North Texas State University, Denton, Texas, 1978.

9. Colowick, Sidney P. and Nathan O. Kaplan, Methods in Enzymology. Vol I, p. 76. 1955. Academic Press, London and New York.

10. Downey, W. K. and P. Andrews. 1965. Gel filtration applied to the study of lipases and other esterases. Biochemistry Journal. 94:642-650,

11. Herndon, William C. 1967. The Structure of Choleic Acids. Journal of Chemical Education. 44:724-728.

12. Hofmann, Alan F. and Donald M. Small. 1967. Detergent properties of bile salts: correlation with physiological function. Annual Review of Medicine. 18:333-376.

13. Hofstee, B. H. J. 1973. Hydrophobic affinity chromatography of proteins. Analytical Biochemistry. 52:430-448.

14. Hofstee, B. H. J. 1973. Protein binding by agarose carrying hydrophobic groups in conjunction which charges. Biochemical and Biophysical Research Communications. 50:751-757.

15. Horiuti, Yosifumi and Sigeyuki Imamura. 1977. Purification of lipase from Chromobacterium viscosum by chromatography on palmitoyl cellulose. Journal of Biochemistry. 81:1639-1649.

16. Kosugi, Yoshitsugu and Hideo Suzuki. 1976. Affinity chromatography of lipase with hydrophobic ligands coupled to cyanogen bromide-activated agarose. Journal of Lipid Research. 17:307-313.

17. Kosugi, Yoshitsugu and Hideo Suzuki. 1974. Affinity chromatography studies on the adsorption of lipase on aliphatic-amine-Sepharose 4B. Journal of Fermentation Technology. 52:577-582.

18. Lawrence, R. C., T. F. Fryer, and B. Reiter. 1967. The production and characterization of lipases from a micrococcus and a pseudomonad. Journal of General Microbiology. 48: 401-418.

19. Levin, Samuel J., C. G. Johnston, and A. J. Boyle, 1961. Spectrophotometric determination of several bile acids as conjugates: extraction with ethyl acetate. Analytical Chemistry. 33:1407-1411.

20. Little, Clive, Bjorg Aurebekk, and Anne-Brit Otnaess. 1975. Purification by affinity chromatography of phospholipase C from Bacillus cereus. FEBS Letters. 52:175-179.

21. Liu, Pinghiu V. 1974. Extracellular toxins of Pseudomonas aeruginosa. Journal of Infectious Deiseases. 130:s94-s99.

22. Liu, Pinghiu V. 1966. The roles of various fractions of Pseudomonas aeruginosa in its pathogenesis. III. Identity of the lethal toxins produced in vitro and in vivo. Journal of Infectious Diseases. 116:139-147.

23. Liu, Pinghiu V. and Charles B. Mercer. 1963. Growth, toxigenicity and virulence of Pseudomonas aeruginosa. The Journal of Hygiene. 61:485-491.

24. Methods in Microbiology. 1969. Vo. 3. Pt. B. Page 145. Edited by J. R. Norris and D. W. Ribbons. Academic Press. London and New York.

25. Morihara, Kazuyuki and Mitsuo Ebata. 1967. Comparison of substrate specificities of a thermostable bacterial elastase. The Journal of Biochemistry. 61:149-151.

26. Mourey, A. 1973. Etude de la lipolyse dans quelques sols forestiers et caractères d'une préporation enzymatique d'origine bactérienne. Thèse de Doctorat de spécialité en biologie végétale, Université de Nancy, France (as sited by Breuil and Kushner [6]).

27. Narasaki, Teiti, Gakuzo Tamura, and Kei Arima. 1968. Studies on the lipoprotein lipases of microorganisms. Part II. Effects of culture conditions on the production of lipoprotein lipases by Pseudomonas sp. M-12-33. Agricultural and Biological Chemistry. 32:1453-1457.

28. Nilsson-Ehle, Peter and Michael C. Schotz. 1976. A stable, radioactive substrate emulsion for assay of lipoprotein lipase. Journal of Lipid Research. 17:536-541.

29. Nord, C. E., L. O. Sjoberg, T. Wadstrom, and B. Wretlind. Characterization of three aeromonas and nine pseudomonas species by extracellular enzymes and haemolysins. Medical Microbiology and Immunology. 161:79-87.

30. Pablo, Gary, Alson Hammons, Sara Bradley, and James E. Fulton. 1974. Characteristics of the extracellular lipases from Corynebacterium acnes and Staphylococcus epidermidis. The Journal of Investigative Dermatology. 63:231-238.

31. Shaltiel, Shmuel and Zvi Er-El. 1973. Hydrophobic chromatography: use for purification of glycogen synthetase. Proceedings National Academy of Science, U. s. A. 70:778-781.

32. Sugiura. Mamoru and Tsutomu Oikawa. 1977. Physiochemical properties of a lipase from Pseudomonas fluoresens. Biochimica et Biophysica Acta. 489:262-268.

33. Tsuzurahara, Kei, Takaharu Hiramatsu, and Shigeyuki Takeyama. 1974. Biphasic effect of taurocholate on lipase activity in the presence of lecithin. Journal of Biochemistry. 75:663-665.

34. Yon, R. J. 1972. Chromatography of lipophilic proteins on adsorbents containing mixed hydrophobic and ionic groups. Biochemical Journal. 126:765-767.

35. Wood, Robert E. 1976. Pseudomonas: the compromised host. Hospital Practice. 91-100.