AN ASSAY METHOD FOR DETERMINING EXTRA-CELLULAR LIPASES FROM PSEUDOMONAS AERUGINOSA

THESIS

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By

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The applicability of an isotopically labelled assay system to determine the lipase production in Pseudomonas aeruginosa was evaluated. Supernatant from cultures of Pseudomonas aeruginosa grown in a medium containing olive oil was incubated with a substrate containing labelled trioleate. Fatty acids were isolated by means of a liquid-liquid partition system. Enzyme activity was determined by measuring the amounts of free fatty acid by liquid scintillation counting. Findings indicate that the isotopically-labelled, liquid-liquid partitioning assay is reliable, sensitive and adaptable to rapid assay conditions. It was also determined that different strains of Pseudomonas aeruginosa produce varying amounts of lipase. Partial purification of supernatant by gel filtration produced two protein peaks showing enzymatic activity.

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CHAPTER I

INTRODUCTION

The widespread use of antibiotics has brought many kinds of infections under control in the compromised patient. In particular, the use of these drugs has reduced the threat of highly invasive streptococcal, pneumococcal and, to a more limited extent, staphylococcal infections. As these virulent organisms have been brought under better control, antibiotic-resistant, opportunistic organisms have begun to replace them as the major threat to the compromised patient. One of these opportunistic bacteria, Pseudomonas aeruginosa, has become the predominant species causing secondary infections in patients suffering from trauma, burns, and certain underlying debilitating diseases (9). Because this organism is widespread in the environment and is resistant to a large number of the commonly used antibiotics, sepsis due to pseudomonas has been encountered with increasing frequency and will most likely continue to be a threat to the debilitated individual (4, 9, 10, 23, 25).

Studies performed in other laboratories (5, 11, 12) have indicated that the extent to which P. aeruginosa can establish an infection depends on the pathogenic properties of the particular strain as well as on the degree of susceptibility

of the host. Extracellular products of P. aeruginosa with possible significance in the pathogenic process include proteases, collagenases, a hemolysin, an elastase, and a lipase (14, 17). The lipase has not received much attention and its contribution to virulence of this species is not clear.

Evaluation of the role of an enzyme in the pathologic process often requires a rapid and sensitive method for enzyme quantitation. Methods presently available for detection of lipase production do not fulfill the requirements for this type of quantitation. Most assay methods are variations of two basic techniques. The first is the diffusion plate technique, in which the enzyme is allowed to diffuse into the medium containing a triglyceride. Fatty acids cleaved off the glycerides form ring-shaped zones of diffusion when precipitated by copper sulfate or other precipitating agents. This system can be used either by allowing the organism to grow on the plate directly or by adding enzyme preparations to wells cut in the agar (8, 19, 21, 22). Large numbers of isolates can be screened for lipase activity, utilizing the medium for growth as well as for enzyme detection. However, the method is not quantitative, and many strains producing lipase of low specific activity may be overlooked. The second technique for quantification of microbial lipases is based on titration of the free fatty acids released from the breakdown of triglyceride (13). In this procedure the enzyme and the substrate mixture are incubated and the fatty

acids released are titrated with an alkaline solution of known strength. Although the titration procedure yields precise results, it is not easily adapted to screening a large number of organisms.

I undertook the development of a technique for the detection of lipase activity which would represent increased sensitivity and specificity over similar methods described in the literature, which is appropriate for use in evaluating the role of the enzyme in the pathologic process.

Belfrage et al. (2) reported on a lipase assay procedure that is based on a bilayer separation of oleic acid from trioleate. In the study reported here, I utilized a variation of Belfrage's technique combined with the use of specifically-labelled trioleate to quantitate extracellular lipases. Isotopically-labelled trioleate increases the sensitivity of the assay since minute quantities of the label can be measured accurately. With this increased sensitivity, the use of the bilayer system allows both the reaction products and unreacted substrate to be quantitated, thus increasing the precision of the assay by providing an internal standard.

The purpose of this study was to determine the feasibility of using the isotopically-labelled bilayer assay system for the measurement of lipase activity of microbial origin. This report also includes preliminary data on some of the properties of the lipolytic enzyme produced by Pseudomonas aeruginosa.

CHAPTER II

MATERIALS AND METHODS

Organisms and Culture Conditions

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 (1) and was used as the principal source of the enzyme for the development of the assay system.

Strains of P. aeruginosa that were used for testing the efficiency of the assay were obtained from clinical specimens and from the stock culture collection of the Department of Biological Sciences at North Texas State University. The majority of organisms isolated from clinical sources were supplied through the courtesy of Dr. Joe Bass, of the University of Texas Health Science Center in San Antonio and Dr. Charles Baxter, University of Texas Health Science Center at Dallas, and Director of the Parkland Hospital Burn Unit. Collection and isolation procedures were those of the contributing hospitals. While all organisms were designated as P. aeruginosa by the hospitals, identity of the strains was confirmed using gram stain morphology, multimedia tubes (Oxi/Ferm tube; Roche Diagnostics), and other criteria listed in Table I. Media were either commercially prepared (Gibco

TABLE I

CHARACTERISTICS USED TO CONFIRM THE ORGANISMS AS PSEUDOMONAS AERUGINOSA

Characteristic	Reaction*
Gram Reaction	Negative
Oxidative or fermentative utilization of glucose	Oxidative
Glucose	A
Catalase	+
Spores	-
Indole	-
Methyl red	-
Xylose	A
Maltose	-
Citrate, Simmons	+
Nitrate to Nitrogen Gas	+
Lysine Decarboxylase	-
Arginine Dihydrolase	+
Ornithine Decarboxylase	-
Pyocyanin Pigment	+/-
Indophenol Oxidase	+

* + = Positive,
- = Negative or no growth,
A = acid production.

Diagnostics) or prepared from dehydrated ingredients (Difco). Stock cultures were grown at 37°C for 18 to 24 hours or until there was adequate growth. All cultures were maintained at 4°C on tryptic soy agar and were subcultured at monthly intervals.

When comparing lipase production among strains, the organisms were grown in 250-milliliter side-arm flasks. The flasks contained 50 milliliters of basal medium, consisting of one percent peptone (Difco) and one percent substrate emulsion. This substrate emulsion was composed of one volume of olive oil (Pompeian) and nine volumes of one percent (W/V) polyvinyl alcohol in water (Matheson Coleman, and Mellman). This mixture was sonicated on a Model W 185D Cell Disruptor (Branson Sonic Power Co.) at 50 watts for 15 seconds to give an emulsion which remained stable for several hours at room temperature. Outdated blood serum obtained from the blood bank of Wadley Institutes of Molecular Medicine was heated to 60°C for thirty minutes to destroy endogenous lipolytic activity. The heated serum was then added to the substrate emulsion in the ratio of one volume emulsion to nine volumes blood serum. To allow the serum and oil emulsion to stabilize, this mixture was then preincubated for 30 minutes at 37°C before use. The inocula consisted of 0.5 milliliter (one percent) of an 18-hour culture grown in trypicase soy broth and diluted with the same broth to a turbidity of 100 Klett units. The turbidity of the culture was measured on

a Klett-Summerson Photoelectric colorimeter using a green number 54 filter (520-580 nm. with maximum transmission at 540 nm.). The cultures were incubated at 37°C for 10 hours on a gyrotary shaker at 100 RPM (stroke of one inch; New Brunswick Scientific Co.). The cell-free supernatant obtained by centrifugation of the culture for fifteen minutes on a Sorvall RC2-B centrifuge at 13,000 x G was used as the crude enzyme preparation.

Determination of Lipase Activity

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)H^3]-oleate was used as the tracer substance. The labelled trioleate was obtained from Amersham/Searle Corporation (99% radiopurity as determined by thin layer chromatography according to the manufacturer's specifications). The "assay substrate" emulsion was prepared similarly to the growth substrate emulsion, except that ten microliters of labelled trioleate per milliliter of substrate were added to the mixture before sonication.

One hundred microliters of the assay substrate emulsion and one hundred microliters of ammonium hydroxide/ammonium chloride buffer (0.2M; pH 8.3) were placed in 20 X 150 millimeter screw cap tubes. The contents of the tubes were equilibrated at 37°C in a rotary water bath (New Brunswick),

and the reaction was started by the addition of two hundred microliters of enzyme preparation. Incubations were for two hours unless otherwise stated.

The liquid-liquid partition system for the separation of fatty acids from glycerides was a modification of a technique developed by Belfrage (2). After incubation, the reaction was stopped by the addition of 6.50 milliliters of a mixture consisting of methanol, chloroform, and n-heptane (1.41:1.25:1;v/v/v). The bilayer system was formed by the addition of 2.1 milliliters of potassium carbonate buffer (0.2M; pH 10). The use of this liquid-liquid partitioning system along with specifically labelled glycerol trioleate allows for the separation and quantification of both unreacted substrate and the reaction products of the lipase reactions.

The radiolabelled fatty acids were determined directly by liquid scintillation counting. Radio assays were run on one-milliliter aliquots of the top, polar layer containing the free fatty acids, and on one-milliliter aliquots of the bottom organic layer containing the unreacted triglycerides. Twenty milliliters of scintillation fluid were added to each aliquot. 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. The final mixture contained 25 percent Triton X-100 as a solubilizing agent in toluene. Samples were counted on a Beckman Scintillation counter Model LS-250.

In certain experiments, the ionic strength of the reacting mixture was increased by the addition of NaCl. The NaCl was added to the ammonium chloride/ammonium hydroxide buffer and the pH readjusted to 8.3, using 0.2 N NaOH or 0.2 N HCl as needed. The assay procedure then employed deviated from that normally used in that only one hundred microliters of enzyme preparation were used while two hundred microliters of buffer containing NaCl were used. All other parameters remained as before.

The percentage hydrolysis of the substrate was determined according to the formula:

((FA x 1/.76)/(FA + TG x 2.63 x 1.20)) x 100 = Percent Hydrolysis.

FA — Net CPM of the upper phase containing the fatty acids

1/.76 — The partitioning coefficient of the fatty acids between the layers as determined by Nilsson-Ehle et al. (16)

TG — Net CPM of the lower phase containing the unreacted triglycerides

2.63 — The quenching correction between phases using stated scintillation fluid determined empirically. (15)

1.20 — The phase volume correction as determined by Belfrage (2).

For comparison between strains, enzyme units were defined as the percentage hydrolysis per hour under stated conditions. All determinations were performed in triplicate unless otherwise indicated.

Purification of P. aeruginosa Lipase

For purification of the enzyme, supernatant from 500 milliliters of basal media was obtained as described above. The supernatant was placed in an ultrafiltration concentrator containing an Amicon Diaflo ultrafiltration membrane with an exclusion limit of greater than 50,000 molecular weight. A portion of the protein in the concentrate was then precipitated using ammonium sulfate in a final concentration of 75 percent saturation (6). The resulting precipitate was dissolved in a minimum amount (≈10 mls) of phosphate buffer (0.2M; pH 8.3) and if needed, it was vacuum concentrated to a final volume of ten milliliters.

Concentrates of culture supernatants were fractionated on a gel filtration column. In preparing the column, twenty grams of dry Sephadex G-200 (Pharmacia Fine Chemicals, Inc.) were placed in five hundred milliliters of phosphate buffer (0.2M; pH 8.3) and allowed to swell overnight at $4^{o}C$. The swollen Sephadex was then de-aerated under partial vacuum and poured into a 60 X 2.5 centimeter column. The final height of the gel was fifty centimeters. The flow rate for the column was approximately fifteen milliliters per hour. The column was allowed to equilibrate for at least twenty-four hours with the same buffer before a 2.5-ml sample was placed on the column. Elution was with the same phosphate buffer. Two-ml fractions were collected by an automatic fraction

collector (ISCO; Model 1200 PMP). The protein content of the fractions was estimated by absorbance at 280 nm. in a Spectronic 210 UV spectrophotometer (Bausch and Lomb). Selected fractions were tested for lipase activity.

CHAPTER III

RESULTS

To ascertain the applicability of the radioactive tracer-bilayer system as an assay, the linearity of the lipolytic reaction with respect to time was determined. Cell-free supernatant obtained from a ten-hour culture of Pseudomonas aeruginosa (19154) was used as the enzyme source. The reaction was stopped at intervals indicated by the data points in Figure 1. Assays were run in triplicate, with the brackets indicating standard deviation. As shown in Figure 1, the rate of fatty acid release was linear at 37°C for at least four hours under the described conditions.

To establish the extent to which hydrolysis was linear with respect to enzyme concentration, an ammonium sulfate concentrate of a cell-free supernatant from a ten-hour culture of P. aeruginosa (19154) was used. Dilutions were made and assayed in the prescribed manner. The resulting curve is linear over the normal range of the assay as can be seen in Figure 2. A regression coefficient of 0.998 was computed for the region of the curve from the origin through 27 percent hydrolysis.

To determine the influence of the blood serum as a part of the basal media, two flasks were inoculated with P.

Fig. 1--Relation of enzyme activity measured under standard conditions to incubation time. A cell-free supernatant obtained from a ten hour culture of Pseudomonas aeruginosa ATCC 19154 was used as enzyme source. The reaction was stopped and assayed at the indicated intervals using the isotopically-labeled bilayer system. Brackets indicate standard deviation among triplicate samples.

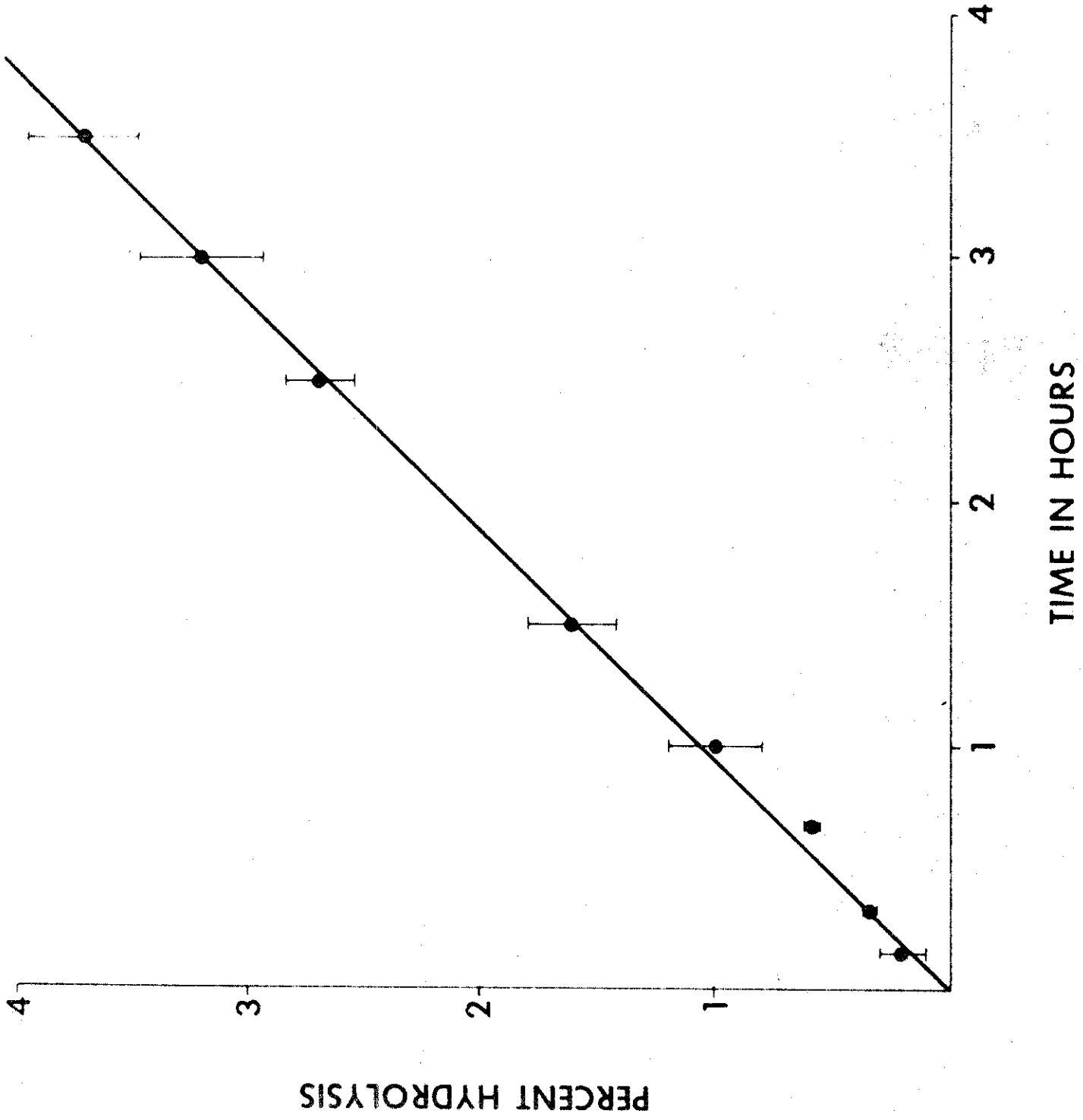
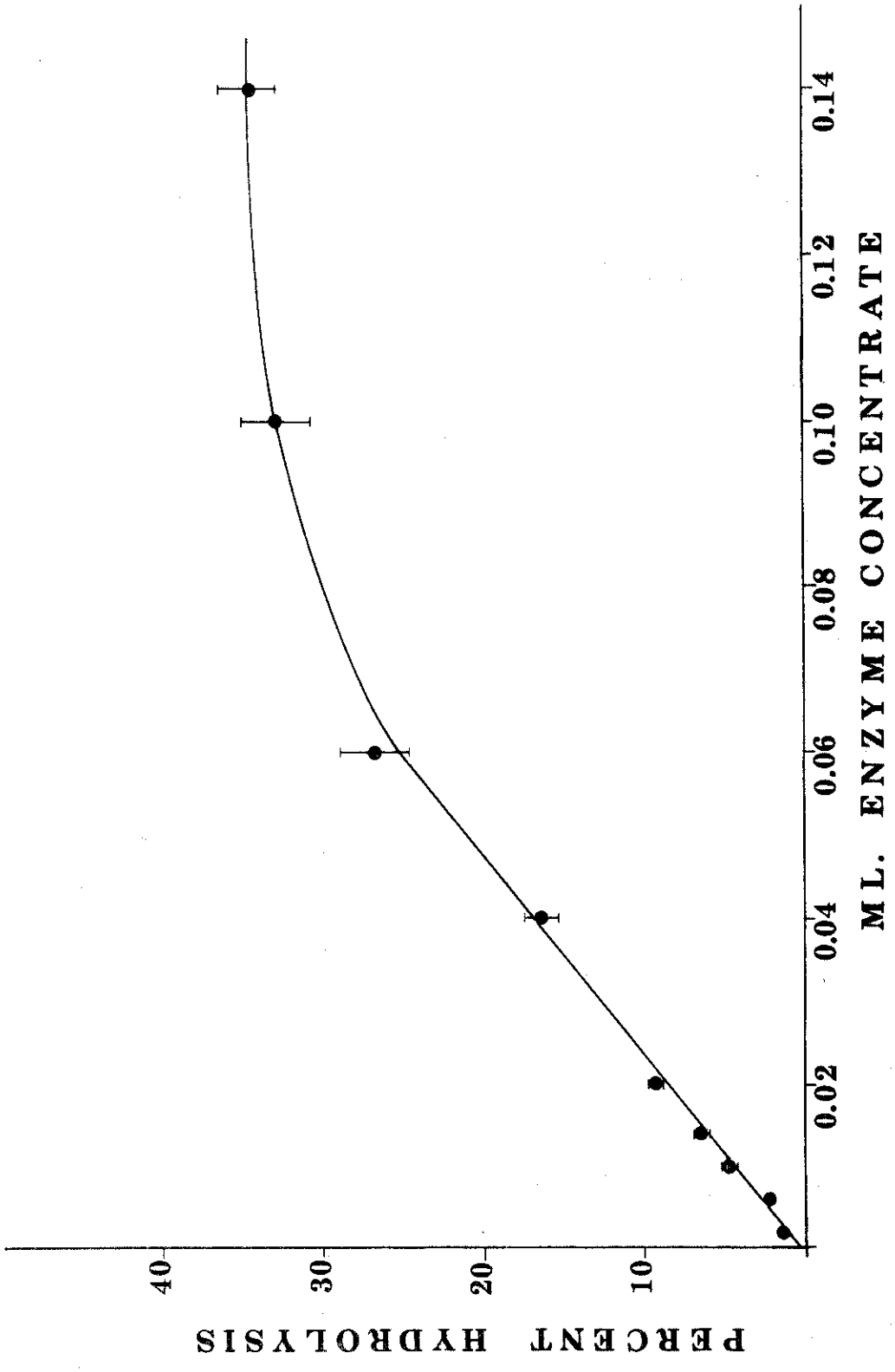

PERCENT HYDROLYSIS
1
2
3
4
TIME IN HOURS
1
2
3
4

Fig. 2--Relation of enzyme activity under standard conditions to enzyme concentration. A cell-free concentrated supernatant from a 10 hour-culture of Pseudomonas aeruginosa ATCC 19154 was used as the enzyme source. The reaction was stopped and assayed after a two hour incubation period at 37°C.

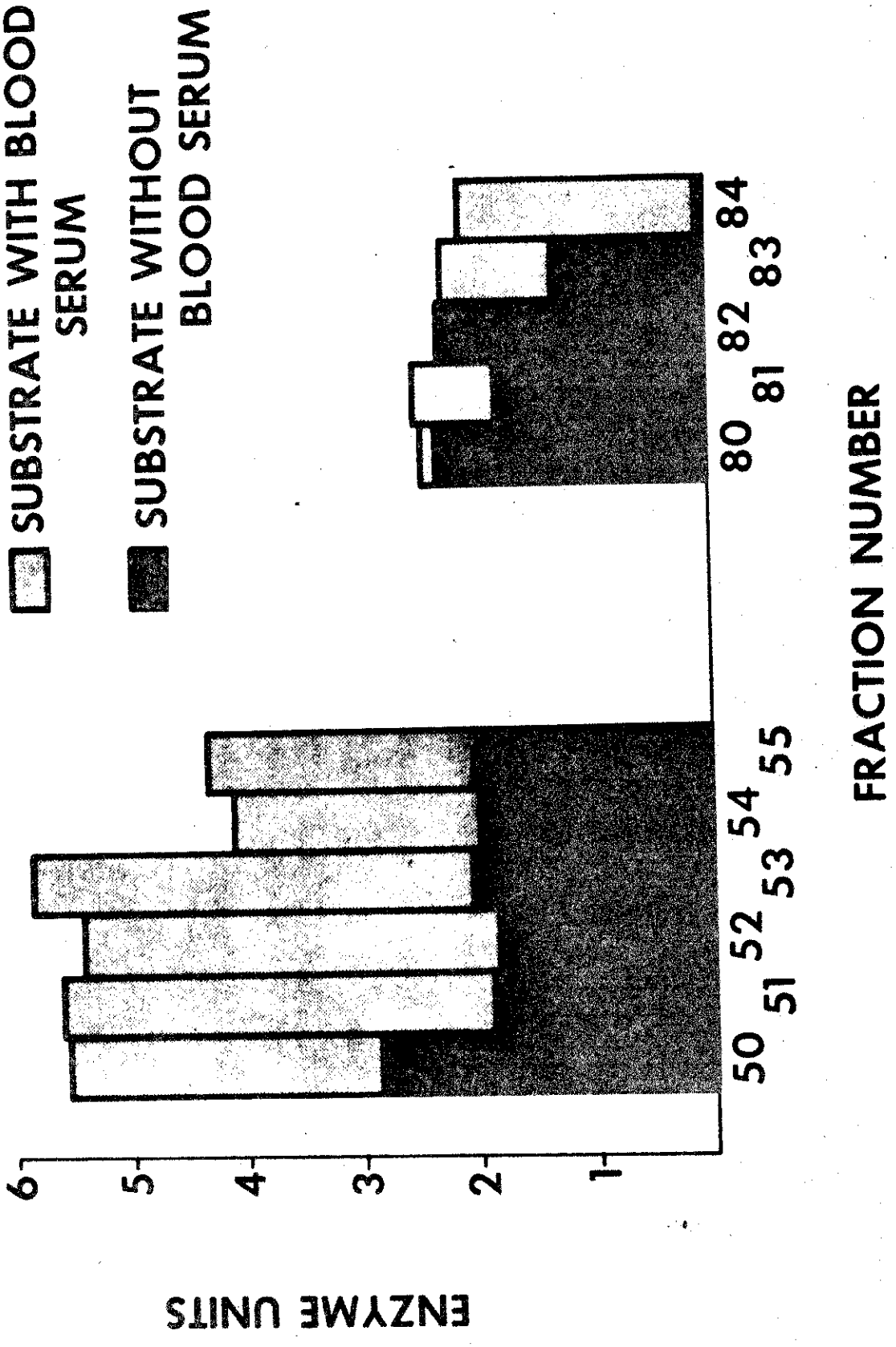

PERCENT HYDROLYSIS
10
20
30
40
0.02
0.04
0.06
0.08
0.10
0.12
0.14
ML. ENZYME CONCENTRATE

aeruginosa (19154) and grown under identical conditions. The first flask contained basal medium containing heated blood serum. The second flask contained identical media, except that the portion of the medium that contained the blood serum was replaced with an equal volume of peptone water. In both cases, lipase production was observed. The flask containing the blood serum, however, showed visibly greater growth and demonstrated higher lipase values.

To ascertain the effect of the blood serum's presence in the assay mixture, a parallel experiment was also run. Two enzyme assays were run on each flask obtained in the previous experiment using the cell-free supernatants as enzyme sources. In the first assay, blood serum was used as a component of the substrate emulsion, while in the second assay, the blood serum was replaced with peptone water. The assay substrate containing blood serum showed consistently higher hydrolysis values. The same phenomenon observed when partially purified enzyme preparations were tested using both assay substrates, Figure 3.

Different concentrations of NaCl were tested to determine the effects of varying ionic strength on the reaction. The enzyme preparation used was from a five-hundred-milliliter concentrate of P. aeruginosa 19154 supernate as described previously. Enzyme concentrate was used to increase the quantity of enzyme used per volume of modified assay

Fig. 3--Relation of enzyme activity measured under standard conditions to the incorporation of heated blood plasma as a part of the assay mixture.


SUBSTRATE WITH BLOOD SERUM
SUBSTRATE WITHOUT BLOOD SERUM
ENZYME UNITS
6
5
4
3
2
1
50
51
52
53
54
55
80
81
82
83
84
FRACTION NUMBER

mixture as described in Material and Methods. The reaction was stopped and assayed as previously described, with the results as depicted in Figure 4.

Lipase activity was enhanced by low concentrations of NaCl. Beyond 0.5M NaCl, however, NaCl was inhibitory. Virtually no enzyme activity at 2.0M NaCl.

In order to assess the effect of the age of the culture on enzyme activity, culture supernatants of three strains of P. aeruginosa; ATCC 19154, 10197 and 27853 were tested for enzyme activity at intervals through a twenty-four-hour growth period. Unbuffered basal medium in side-arm flasks was inoculated with a one percent standardized inoculum of the respective organism and incubated at 37°C as previously described. At two-hour intervals, turbidity readings were made and one-milliliter aliquots of media were withdrawn from each flask. After centrifugation, the supernatants were checked for pH and assayed for enzyme activity. The quantity of growth as well as the quantity of enzyme production varied between strains, as can be seen in Figure 5, 6, and 7. In each of the three organisms studied, there is a peak in enzyme production at approximately 10-12 hours of growth. These peaks also appear to correspond with fluctuations in the hydrogen ion concentration of the unbuffered media in a range which falls on either side of pH 8.

Fig. 4--The effect of NaCl concentration on lipase activity of Pseudomonas aeruginosa ATCC 19154.

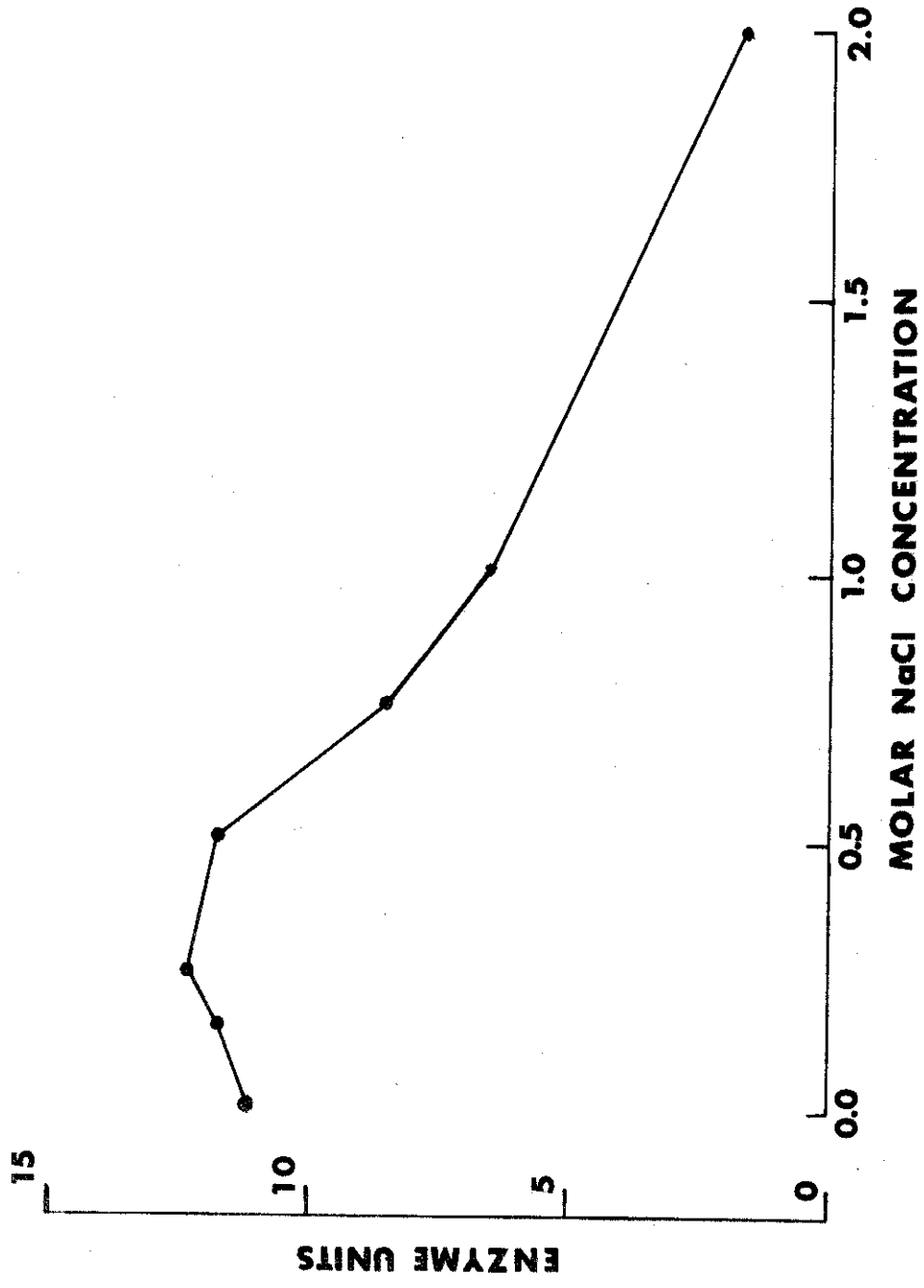

ENZYME UNITS
0
5
10
15
MOLAR NaCl CONCENTRATION
0.0
0.5
1.0
1.5
2.0

Fig. 5--Turbidity, lipase production and pH during 20 hours of growth of Pseudomonas aeruginosa ATCC 19154 in the unbuffered, basal medium. (●-●) Turbidity in Klett units at 540 nm; (▶-▶) pH; (■-■) Lipase enzyme concentration in enzyme units defined as percentage hydrolysis per hour under standard conditions.

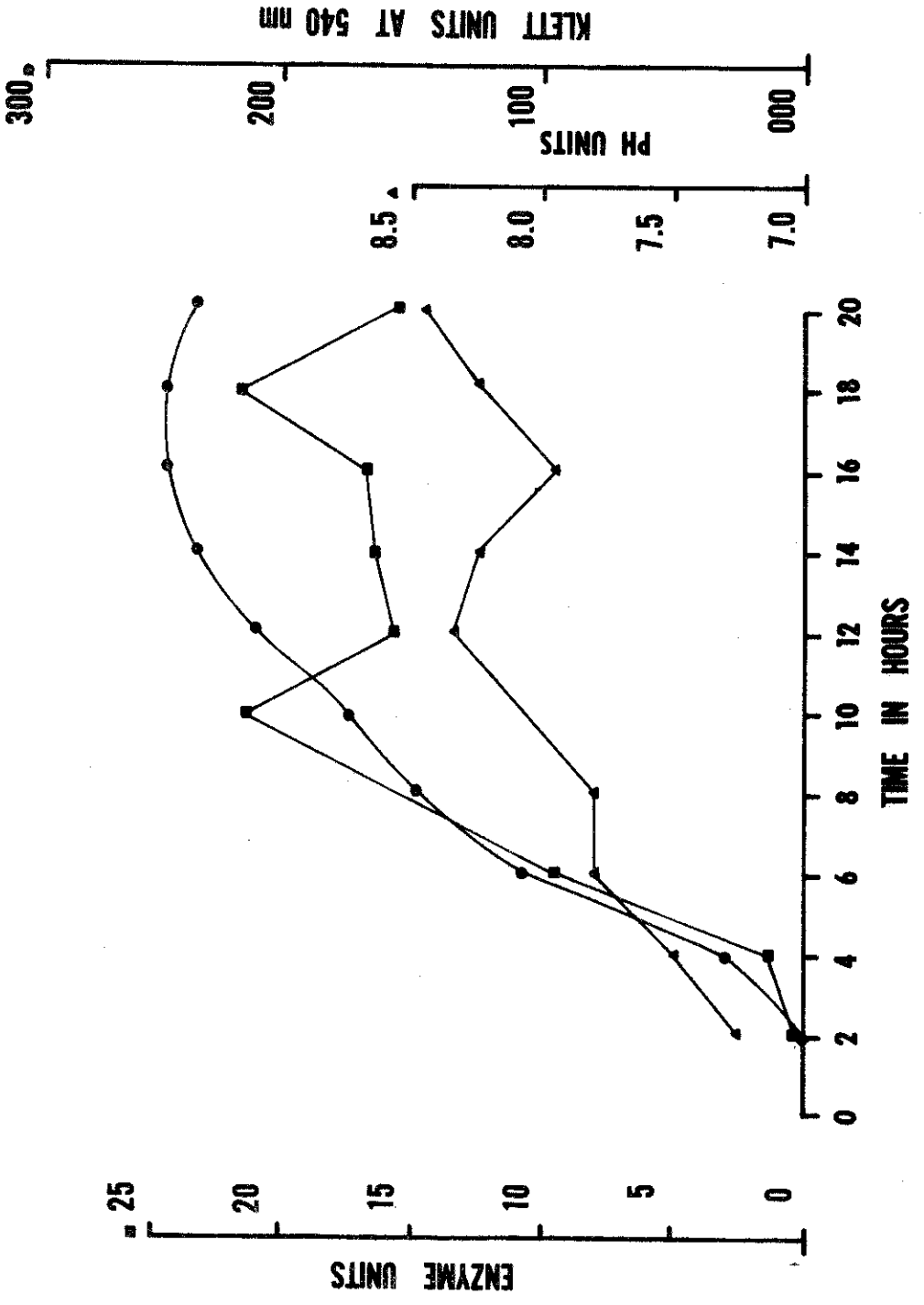

KLETT UNITS AT 540 nm
300
200
100
000
PH UNITS
8.5
8.0
7.5
7.0
TIME IN HOURS
0
2
4
6
8
10
12
14
16
18
20
ENZYME UNITS
25
20
15
10
5
0

Fig. 6--Turbidity, lipase production and pH during 20 hours of growth of Pseudomonas aeruginosa ATCC 27853 in the unbuffered, basal medium. (●-●) Turbidity in Klett units at 540 nm; (▶-▶) pH; (■-■) Lipase enzyme concentration in enzyme units defined as percentage hydrolysis per hour under standard conditions.

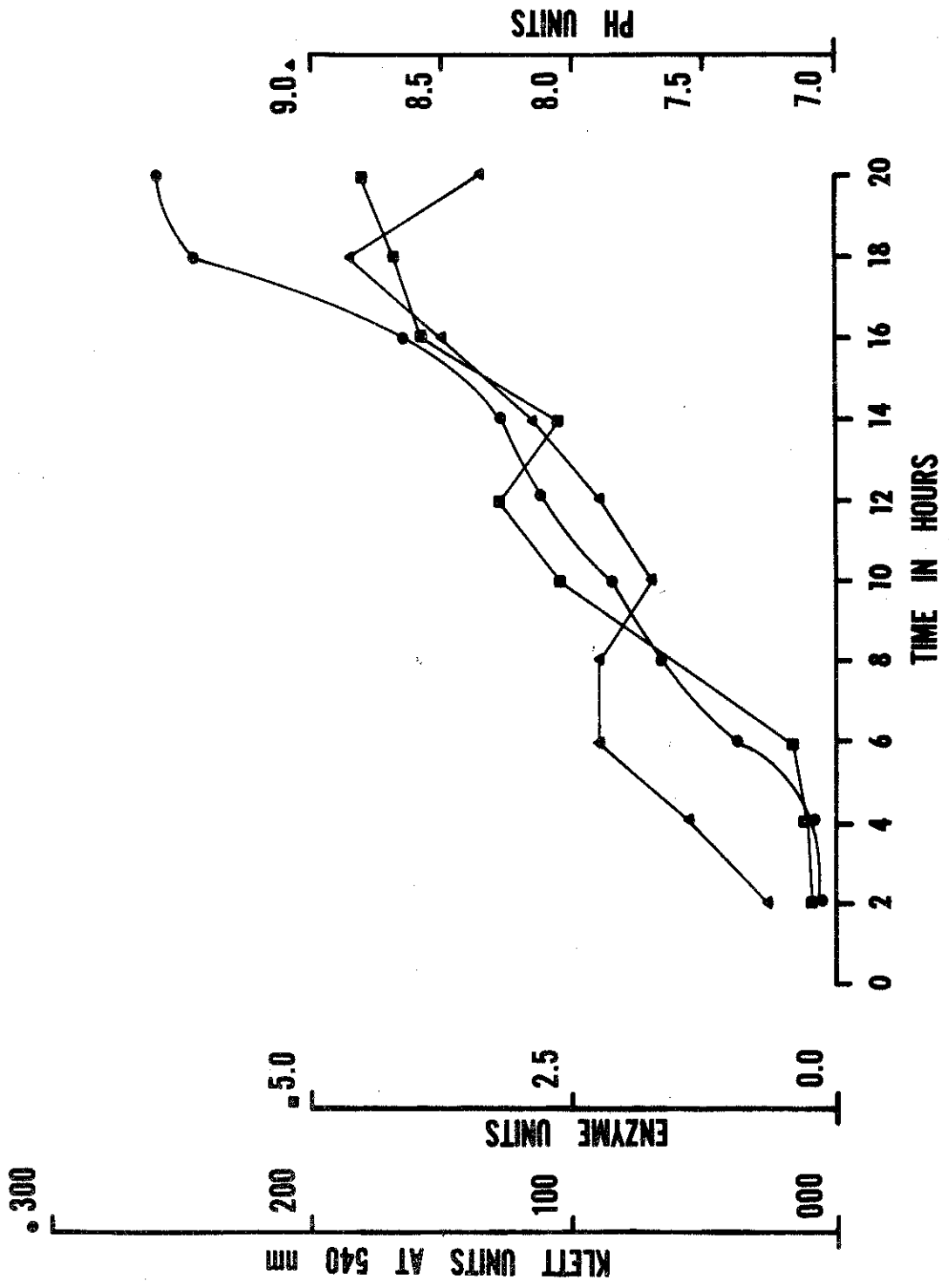
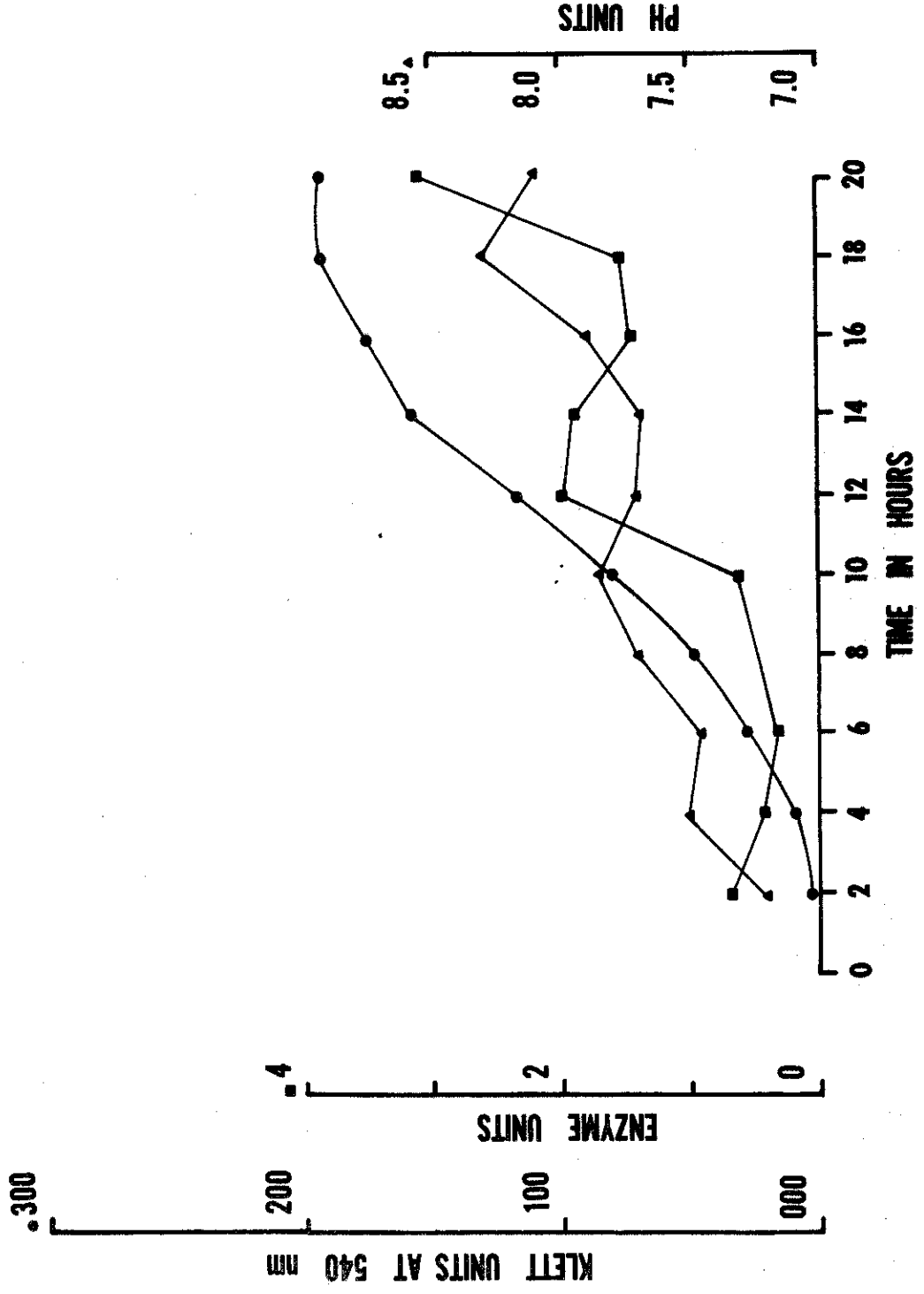

KLETT UNITS AT 540 mμ
300
200
100
000
ENZYME UNITS
5.0
2.5
0.0
PH UNITS
9.0
8.5
8.0
7.5
7.0
TIME IN HOURS
0
2
4
6
8
10
12
14
16
18
20

Fig. 7--Turbidity, lipase production and pH during 20 hours of growth of Pseudomonas aeruginosa ATCC 10197 in the unbuffered, basal medium. (●-●) Turbidity in Klett units at 540 nm; (▶-▶) pH; (■-■) Lipase enzyme concentration in enzyme units defined as percentage hydrolysis per hour under standard conditions.


KLETT UNITS AT 540 nm
300
200
100
000
ENZYME UNITS
4
2
0
TIME IN HOURS
0
2
4
6
8
10
12
14
16
18
20
PH UNITS
8.5
8.0
7.5
7.0

A survey of different strains of P. aeruginosa was undertaken using the assay as described. Cultures were obtained from the American Type Culture Collection, clinical laboratories, and the stock cultures collection held by the Department of Biological Sciences of North Texas State University. All cultures were checked for purity and characterized as stated previously. The results of the survey are depicted in Table II. In all cases, there was enzyme production, although there was a wide variation in the amount produced between cultures. The retesting of selected cultures after having been carried on artificial media also showed variations in enzyme production. The variation was normally in a downward direction, although several exceptions to this trend were noted. A lack of a significant quantity of triglycerides in the Tryptocase soy medium, with a corresponding shutdown of the extra-cellular lipase enzyme system may account for some of this variation.

An attempt was made to partially purify and characterize the enzyme. Cell-free supernatant from a culture of P. aeruginosa 19154 was concentrated as previously described. The concentrate was fractionated on a Sephadex G-200 gel filtration column. As Figure 8 indicates, the protein peak associated with the highest enzymatic activity is the first protein to elute from the column. This is indicative of a substance of high molecular weight. Smaller protein peaks

TABLE II

STRAINS OF PSEUDOMONAS AERUGINOSA TESTED FOR LIPASE PRODUCTION. SELECTED STRAINS WERE RETESTED AFTER HAVING BEEN MAINTAINED ON ARTIFICIAL MEDIA (TRYPTICASE SOY AGAR)

Strain Designation	Isolation Source	Lipase Units First Test	Lipase Units Retest
19154	ATCC[a]	18.70	5.04
9721	ATCC/NTSU[b]	2.01	5.12
27853	ATCC/NTSU	3.95	2.17
10197	ATCC/NTSU	0.27	0.45
1369	NTSU[c]	0.10	0.64
Sputum 2	Clinical[d]	0.33	
Sputum 3	Clinical	1.94	
WD-1	Clinical	0.63	
WD-4	Clinical	0.31	0.62
WD-7	Clinical	1.82	4.43
2177F	Clinical	2.78	0.66
2177S	Clinical	2.29	0.61
SB-1	Clinical	3.52	0.59
SB-2	Clinical	3.87	14.61
SB-3	Clinical	0.41	0.74
SB-4	Clinical	1.53	0.97
SB-5	Clinical	1.50	

TABLE II--Continued

Strain Designation	Isolation Source	Lipase Units First Test	Lipase Units Retest
Blood-1	Clinical	0.64	
P3701	Clinical	0.51	0.78
P3702	Clinical	0.93	
P3703	Clinical	1.01	
P3704	Clinical	1.33	
P3705	Clinical	0.87	0.86
P3706	Clinical	1.46	
P3707	Clinical	0.98	
P3708	Clinical	1.34	
P3709	Clinical	1.49	0.63
P3710	Clinical	0.89	

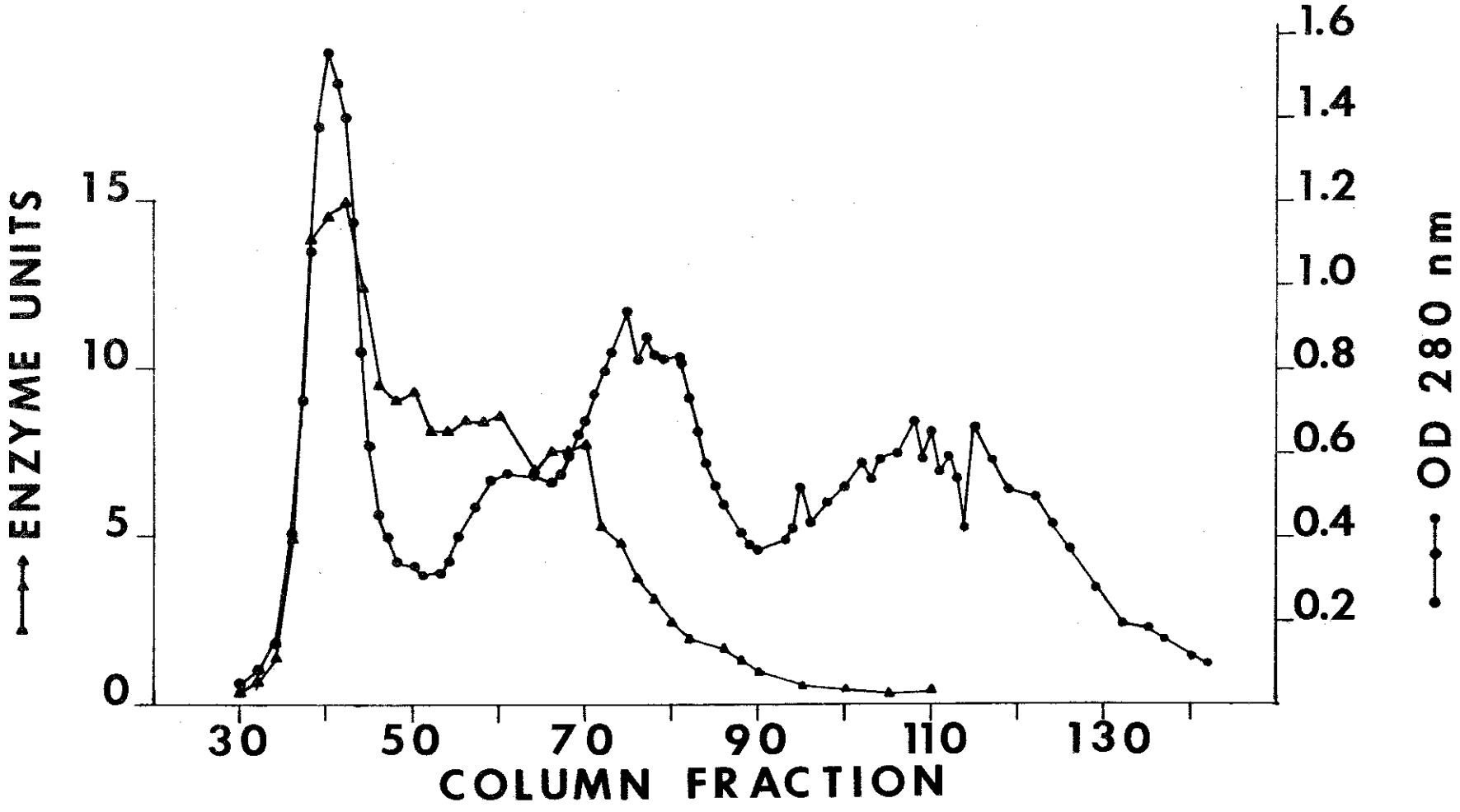
[a]Obtained from the American Type Culture Collection, Rockville, Maryland.

[b]Stock culture held by the Department of Biological Sciences, North Texas State University, but originally obtained from the American Type Culture Collection.

[c]Stock culture held by the Department of Biological Sciences Department, North Texas State University.

[d]Culture obtained from clinical isolates from the University of Texas Health Science Center at San Antonio or University of Texas Health Science Center at Dallas.

Fig. 8--Elution pattern from Sephadex G-200 using concentrated cell-free supernatant from a 10 hour culture of Pseudomonas aeruginosa ATCC 19154.


ENZYME UNITS
15
10
5
0
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
OD 280 nm
30
50
70
90
110
130
COLUMN FRACTION

eluting after the first major peak also showed some enzyme activity. After the second major protein peak eluted, there was a sharp drop in the enzymatic activity of the fractions, however.

CHAPTER IV

DISCUSSION

This work demonstrates the feasibility of the bilayer-radiolabelled lipase assay for use with microbial systems. The assay combines the sensitivity and reproducibility of the tracer assay with the rapidity necessary for the screening of a large number of isolates. It also shows a large range of hydrolysis values over which there is a direct correlation between the percentage of hydrolysis and the amount of enzyme present. The presence of heated blood serum in the emulsion substrate contributed negligible endogenous lipolytic activity and permitted the detection of lipase in cultures weakly lipolytic.

It was found that all the isolates tested have produced some lipase activity. This agrees with other published reports which show that lipolytic activity is a characteristic of P. aeruginosa (17, 18). Other reports have indicated that not all strains produce the enzyme. This may be attributed to the lack of sensitivity of the employed assay. The possibility also exists that certain of the organisms tested in this study were preferentially selected because they produce a lipase enzyme. Organisms other than those

obtained from stock culture collections were clinical specimens where the production of this enzyme may offer an advantage to the organism through increased tissue destruction. Of the isolates obtained from culture collections, the majority were originally obtained from clinical specimens.

Partial purification of the lipolytic enzyme was achieved. There appears to be at least two protein peaks that show enzyme activity after passing through the Sephadex column. Whether these two peaks are the result of two different enzymes or are the result of either the breakdown or aggregation of the original enzyme has not been determined.

As with most exoenzymes, the Pseudomonas aeruginosa lipase complex apparently is rather stable. In concentrated form it had lost very little activity after several day's storage at $4^{o}C$.

The pseudomonas enzyme appears to have several characteristics in common with lipases/lipoprotein lipases derived from other sources. In addition to its similar enzymatic function, both enzymes are enhanced by blood serum and respond similarly to NaCl which stimulates the enzymes at low concentrations and inhibits them at high (above 0.5M) concentrations (7,20). Without further characterizations, the classification of the enzyme as a lipase or a lipoprotein lipase cannot be made.

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