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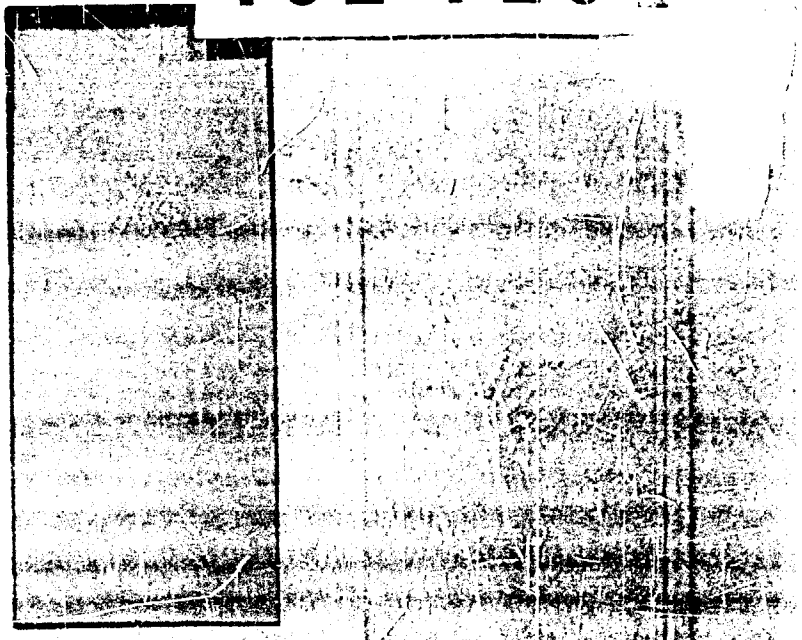

Biochemical Detection Methods for Bacteria and Viruses

Third Quarterly Progress Report

1 January 1963 to 31 March 1963

Prepared by

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Contract DA 18-064-CML-2842

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ABSTRACT

The purpose of this work was to test the feasibility of enzymatic methods for the rapid detection of airborne bacteria and viruses. The effort during the third quarter was devoted to quantitating the microphotofluorimeter, the application of the bacterial esterase to this assay system, and studies on the amplified enzyme system using papain.

The microphotofluorimeter was standardized, and various factors affecting the bacterial esterase were studied and evaluated. Among these were heat denaturation, comparison of other substrates, and salt effects. Thirteen different species of bacteria were examined for esterases to hydrolyze fluorescein diacetate. The enzymes responsible for hydrolysis of phenylacetate and FDA are both universally present in all bacteria, including the anaerobes which were not previously tested.

The possibility of using the amplified enzyme approach for detection was tested using papain. Various activators, including whole and broken bacterial cells, were examined for their ability to activate papain. Because activation by bacteria required large numbers of cells, possible methods of amplifying these effects were explored. A system containing cholinesterase, acetylthiocholine, papain, and CTN was used to demonstrate that the product from one enzyme system can activate a second enzyme. Various methods for applying this approach to detection of bacteria are suggested.

The data demonstrates the potential of the microscope esterase technique and indicates that intensive research should be performed with this system.

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1. INTRODUCTION

This is the third quarterly progress report, submitted in compliance with Contract DA-18-064-CML-2842, on work performed during the period 1 January 1963 to 31 March 1963. The work started in the second quarter, on the amplification techniques for detection, was continued and progress was satisfactory.

In this quarter, the microscope was assembled and modified to measure the fluorescence of fluorescein. After calibration of the instrument to give a linear response to fluorescein concentration, the system was applied to the bacterial esterase system. By employing the microscopic technique, it was possible to follow the enzymatic hydrolysis of fluorescein diacetate (FDA) by bacteria.

In the first quarterly report, the presence of phenylacetate esterase was found to be universal in all bacteria. There was some doubt, however, whether FDA esterase was universally present. In the third quarter, 13 different bacterial species, including two anaerobic organisms, were examined for FDA esterase.

The amplified enzyme system, where a product of one enzyme reaction is used to activate a second enzyme, was also studied during this period. Two forms of inactive papain were investigated. Activation of papain by the organisms per se was tested. Studies on model systems were made to measure the potential of the system.

The results of these studies were interpreted in terms of the ultimate objectives of this program.

2. EXPERIMENTAL WORK

2.1 Background

Work during the first quarterly reporting period demonstrated that the esterase responsible for hydrolysis of phenylacetate was universally present in all the bacteria tested. In the second report, it was demonstrated that fluorescein diacetate (FDA) could be hydrolyzed by bacteria. The conditions for maximal activity of the esterase enzyme in Serratia marcescens were studied; these conditions were employed in the studies throughout this report. It was important to show that FDA esterase occurs universally in all bacteria, and especially in anaerobic bacteria. This was one of the tasks assigned to the present quarter's work. Preliminary work with the microscopic measurement of fluorescence occurring in small droplets was also mentioned in the second quarterly report. Improvements in this technique, and the quantitative measurement of fluorescein concentrations, were one of the primary objectives of the work performed during this reporting period. Another objective for this period was the quantitation of enzymatic hydrolysis of FDA in microdroplets. This latter experiment was necessary to demonstrate the feasibility of using the microscopic technique to follow enzyme reactions.

Another task during the third quarter was to study papain activation as a method of amplifying an enzyme reaction. The possibility that bacteria would activate papain was also to be considered.

2.2 Esterase Studies on the Fluorimeter

2.2.1 Hydrolysis of FDA by Bacteria

Thirteen organisms were selected as representative genera to show that FDA esterase is universal in all bacteria.

Both aerobes and anaerobes were tested. The procedure for growth and collection of the aerobic bacteria was as follows: For preparation of inoculum, trypticase soy broth tubes were inoculated and allowed to incubate for 18 hours at the optimum growth temperature for each of the cultures. One ml of the inoculum was spread on the surface of TS agar in petri plates. The plates were incubated overnight (18 hours). The cells were scraped into cold, distilled H_2O, washed twice with distilled H_2O and suspended 0.05M phosphate buffer, pH 7.0. Cell counts were made in the Petroff-Hauser counting chamber. Appropriate dilutions were made so that the same number of cells was used for each organism tested. The anerobic bacteria were grown 24 hours at 37°C in a beef liver medium, and the cells were collected by centrifugation. The cells were washed and counted as above.

The reaction mixtures contained 5×10^{10} bacteria (0.5 ml); 1.0 ml of 10^{-4}M FDA; 1.0 ml of 0.5M phosphate buffer, pH 7.5; and 0.5 ml water. Controls for nonenzymatic hydrolysis were run as above except that the bacteria were boiled prior to addition to the reaction mixture. Reagent controls, with water substituted for the bacteria, were also included. The reactions were started by the addition of substrate. The mixtures were incubated at 27°C for 15 minutes prior to measurement of fluorescence. The boiled organisms showed no fluorescein above the reagent control, confirming the fact that enzymatic hydrolysis was responsible for the increased fluorescence.

The results in figure 1 indicate that a universal esterase for FDA hydrolysis is present in all the bacteria tested. The

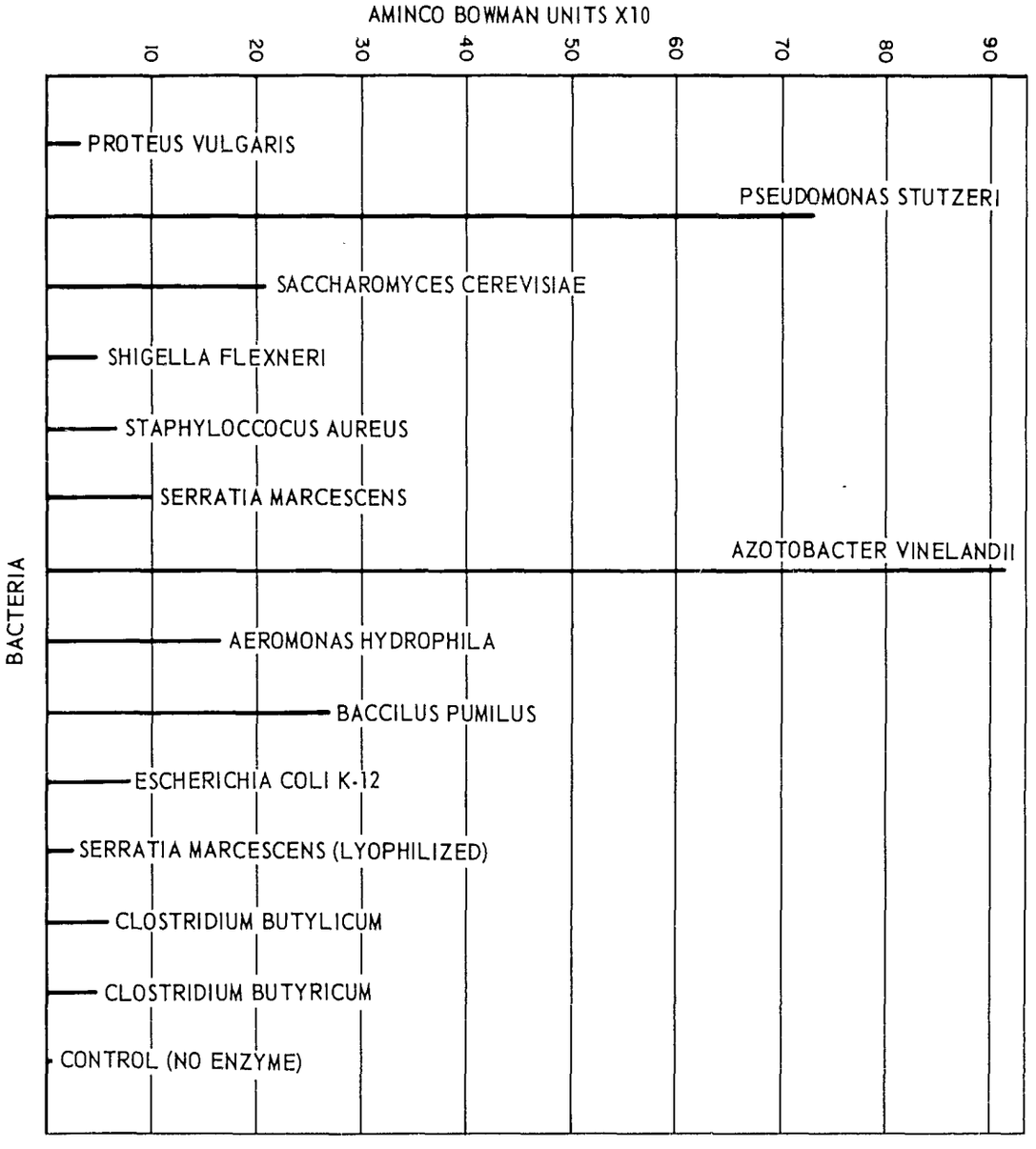

Figure 1. Relative Hydrolysis of FDA by Thirteen Organisms

large differences in total hydrolysis observed in figure 1 are probably caused by the differences in products formed. In the Pseudomonas and Azotobacter, the product is probably fluorescein rather than FMA. However, regardless of the product, the experiment demonstrates the wide distribution of esterase enzymes.

The screening experiment described above was also repeated with cell-free preparations. Results were identical to the whole cell experiment, except the cell-free preparation showed greater activity.

The results obtained with the anaerobic bacteria, Clostridium butylicum and Clostridium butyricum, should be noted because this is the first report to show hydrolysis of esters, especially FDA, by anaerobic bacteria. Relatively low esterase activity is shown in both the strains tested. Additional anaerobes and spores should be tested for ability to hydrolyze FDA as time permits.

2.2.2 Hydrolysis of Various Esters by Esterase

2.2.2.1 Hydrolysis of FDA and Fluorescein Dibutyrate (FDB): FDB, obtained from the Baltimore Biological Laboratory, was tested for activity with lyophilized cells of Serratia marcescens, and compared with the activity on FDA.

The results of the experiment are shown in figure 2.

S. marcescens	1 ml	2 ml	3 ml	4 ml
1×10^{12}	0.5		0.5	
1×10^{-4} M FDA	1.0	1.0		
1×10^{-4} M FDB			1.0	1.0
0.5 M phosphate buffer, pH 7.4	0.5	0.5	0.5	0.5
H_2O	1.0	1.5	1.0	1.5

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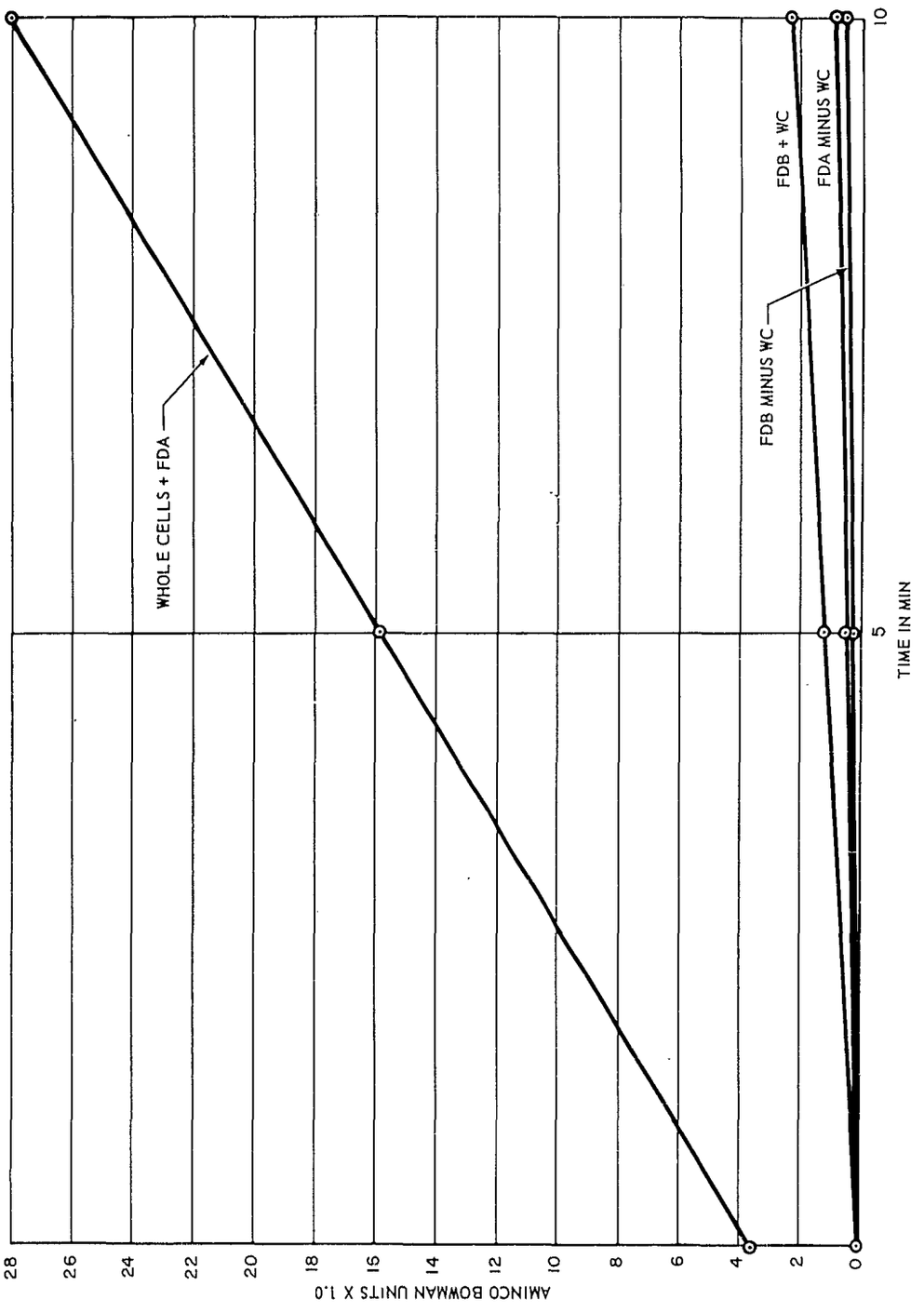

Figure 2. Comparison of Esterase Activity with FDA and Fluorescein Dibutyrate

The reactions were started by addition of substrate and the mixtures were incubated for 1, 5, and 10 minutes at 37° before measurement of fluorescence at 480/520 on the Aminco Bowman in 3.0 ml cuvettes. As the results show, the activity on FDB was less than 10% of the activity observed with the FDA. It appears that longer chain-length esters of fluorescien are less useful for detection than shorter chain lengths. No ester with a shorter chain length than acetate was available and therefore could not be tested. The formate esters of fluorescein should be considered because higher turnover numbers might be realized.

2.2.2.2 Comparison of Rates of Hydrolysis with Two FDA Preparations: Two preparations of FDA were synthesized in Melpar's laboratories, one of which was recrystallized twice and was thought to have a higher purity. The experiment was run, and the data are shown in figure 3.

	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml
Whole cells						
S. marcescens 1 x 10^{12}	0.3	0.3	0.3			
1 x 10^{-4} M FDA (Melpar No. 1)	0.5			0.5		
1 x 10^{-4} M FDA (Melpar No. 2)		0.5			0.5	
0.5 M PO_4 PH 7.0	0.2	0.2	0.2	0.2	0.2	0.2
H_2O				0.3	0.3	0.3

Reactions were initiated with substrate addition and fluorescence measurements were made in 100 µl cuvettes in the Aminco Bowman. Samples were taken at 0, 15, and 30 minutes. The incubation temperature was 27°. The lower activity observed with the No. 1 preparation may be caused by its lower purity.

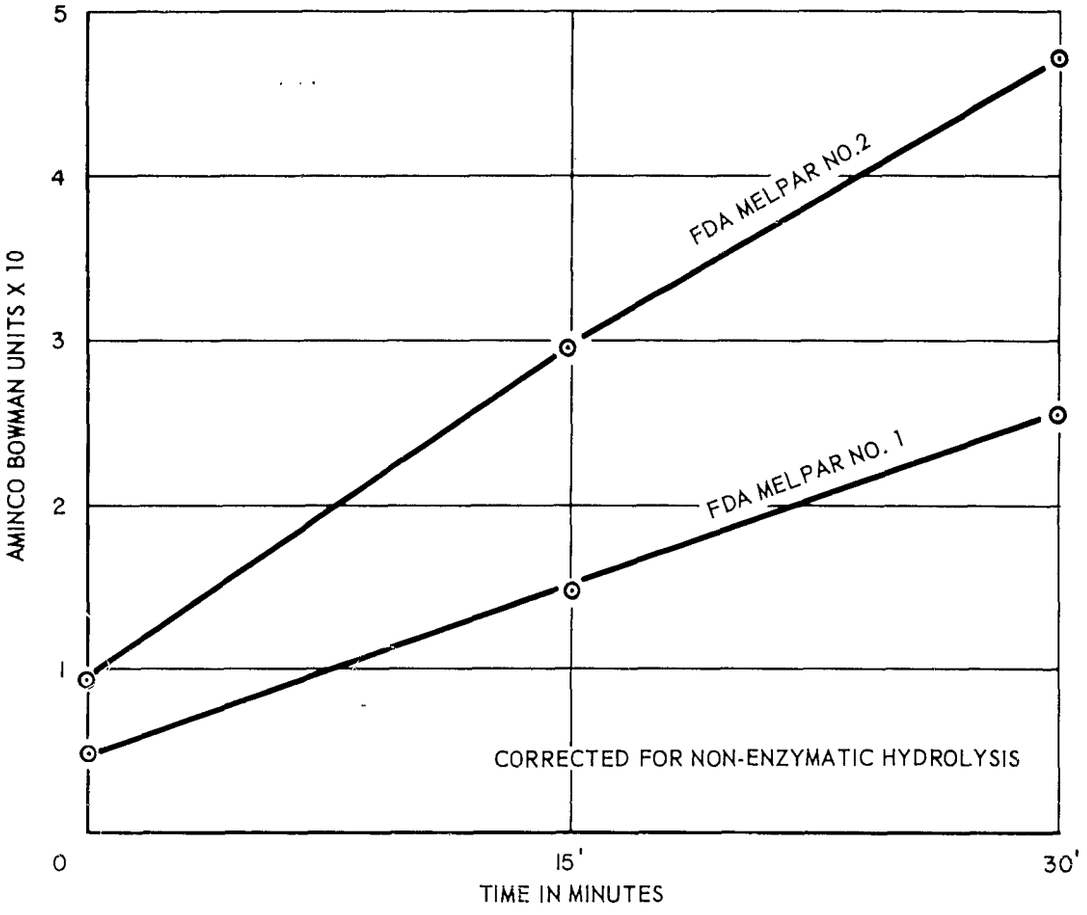

Figure 3. Hydrolysis of Three FDA Preparations

2.2.3 Effect of Heat Denaturation of Bacteria on the Esterase Activity

Because the product of FDA hydrolysis was not known, it seemed possible that two enzymes might be involved in the complete hydrolysis of FDA to fluorescein. Through heat exposure, it was hoped to show partial denaturation, perhaps denaturing one enzyme and leaving the other intact.

The procedure to test this hypothesis was as follows. Suspensions of 2×10^{12} whole cells of Serratia marcescens, suspended in phosphate buffer .01M, pH 7.0, were exposed to temperatures of 55°, 75°, and 100°C for 10 minutes. These samples, along with the untreated cells, were incubated with FDA and FMA. The reaction mixtures were sampled at 0, 15, and 30 minutes and assayed at 27° in 100 µl cuvettes in the Aminco Bowman.

The results are shown in figure 4.

	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	7 ml	8 ml	9 ml	10 ml
2×10^{12} whole cells untreated		0.3					0.3			
whole cells 55°			0.3					0.3		
whole cells 75°				0.3					0.3	
whole cells 100°					0.3					0.3
1×10^{-4} M FDA	0.5	0.5	0.5	0.5	0.5					
1×10^{-4} M FMA						0.5	0.5	0.5	0.5	0.5
M PO_4 pH 7.0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
H_2O	0.3					0.3				

After treatment at 55°, the ability of the enzyme(s) to hydrolyze FMA was only slightly effected, while the ability to hydrolyze

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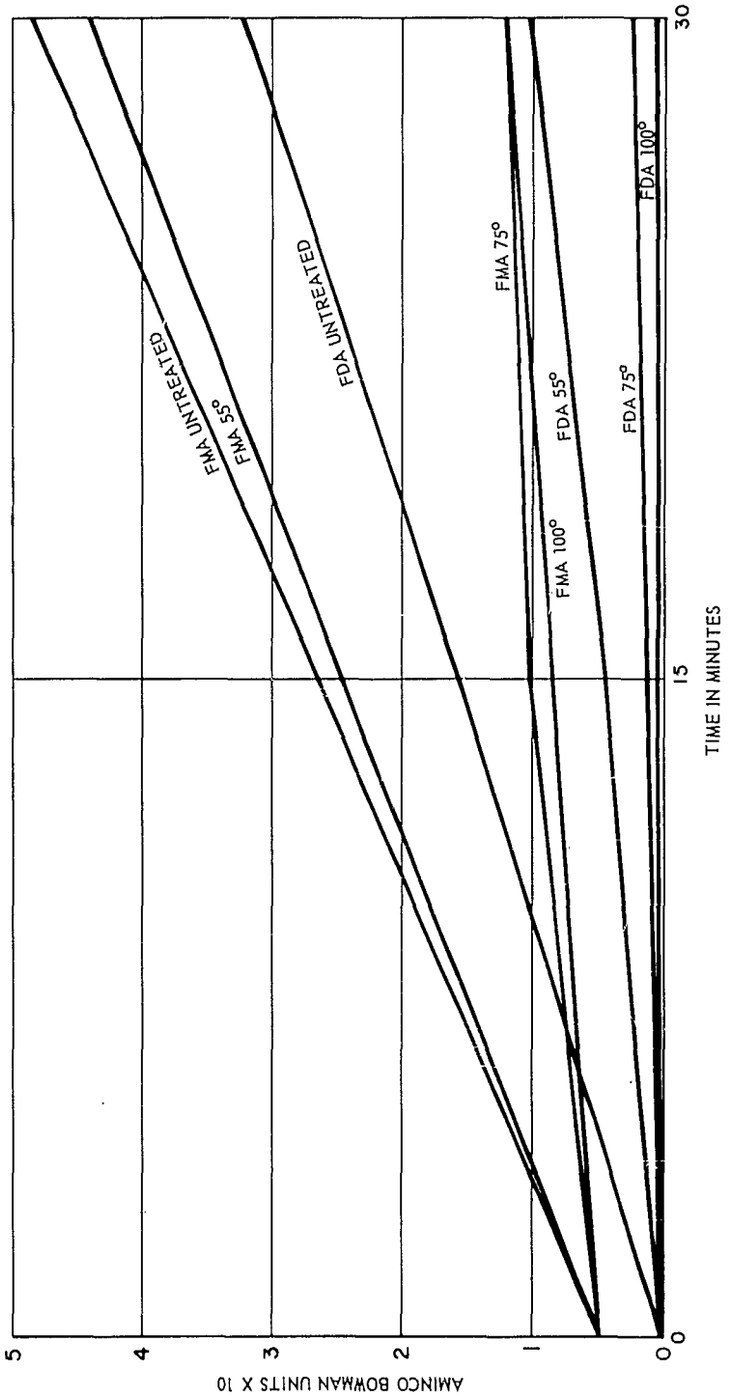

Figure 4. Effect of Heat Treatment on Esterase Activity

FDA was decreased by 75%. The cells exposed to 75° are almost completely inactive with either FMA or FDA. The latter treatment was expected to inactivate the enzymes.

Although these results are only preliminary, it appears that two enzymes may exist and that further study in this area is needed.

2.3 Effect of Salt on Fluorescence

In the second quarter's work, it was found that cells suspended in phosphate buffer exhibited much higher enzyme activity than cells suspended in NaCl. To determine whether this effect was a result of quenching[2] by the chloride or potassium ion or whether this was caused by enzyme inhibition, the following experiment was run. Results are shown in figure 5.

Sample No.	1	2	3	4	5	6	7	8	9	10
0.1 M KCl	0.5					0.5				
0.1 M KBr		0.5					0.5			
0.1 M KI_2			0.5					0.5		
0.1 M NaCl				0.5					0.5	
0.5 M Buffer* pH 7.4	0.5	0.5	0.5	0.5		0.5	0.5	0.5	0.5	0.5
1×10^{-4} M FMA	1.0	1.0	1.0	1.0	1.0					
1×10^{-4} M F						1.0	1.0	1.0	1.0	1.5

*Sorenson's.

The samples were incubated with the appropriate halide for 10 minutes and then compared with controls containing no halide. Measurements were made in the Aminco Bowman photofluorimeter. There was no significant quenching as a result of the halide, as had been reported by Udenfriend.[2] Therefore, the effect must be caused by enzyme inhibition. This would explain the much higher activities of the cells in the absence of these halides.

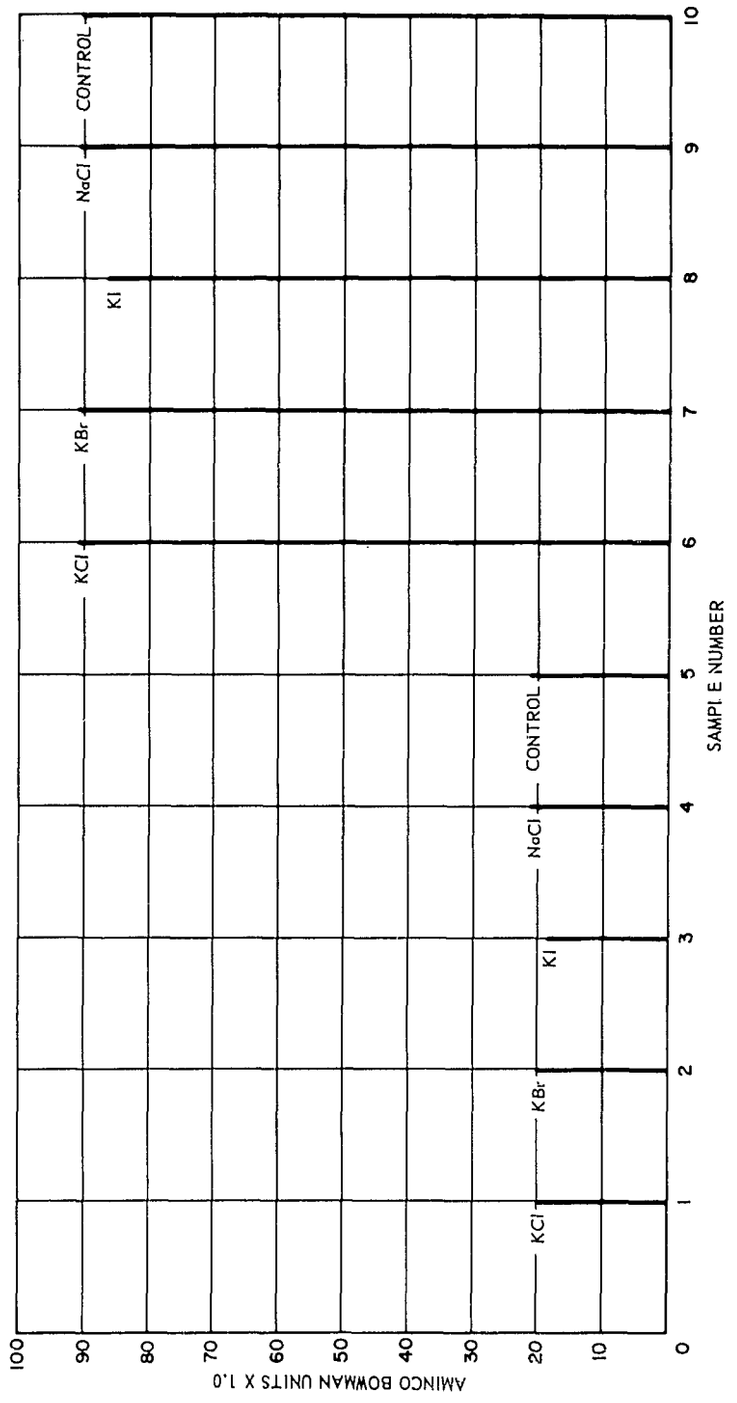

Figure 5. Effect of Salt on Fluoresence of Fluorescein and FMA

2.4 Measurement of Fluorescein on the Microscope

2.4.1 Calculations of Drop Size

Prior to making actual measurements with the microscope, several preliminary calculations had to be made. The range of droplet sizes was obtained from the work of Rotman.[1] Volumes of droplets ranging in diameter from 12-22 microns were calculated using the following formula.[3] $4/3\pi r^3$ = volume of a sphere, where r is expressed in microns ($1\mu = 1 \times 10^{-4}$ cm). The volumes of the following drops were calculated as follows:

$12\mu = 9.0 \times 10^{-10}$ cm^3
$14\mu = 1.41 \times 10^{-9}$ cm^3
$15\mu = 1.69 \times 10^{-9}$ cm^3
$16\mu = 2.15 \times 10^{-9}$ cm^3
$17\mu = 2.58 \times 10^{-9}$ cm^3
$18\mu = 3.06 \times 10^{-9}$ cm^3
$19\mu = 3.60 \times 10^{-9}$ cm^3
$20\mu = 4.2 \times 10^{-9}$ cm^3

The size of the drops in the microscopic field was determined with a Leitz cross-hair eyepiece, reticular calibrated in microns (obtained from Bunton Instrument Co., Washington, D.C.)

2.4.2 Preparation of Microchamber

The preparation of the microchamber and its use are as follows:

1. Dark field slides of uniform thickness are selected and matched to the dark field condensor so that illumination will be constant and the subjective function of condensor focusing eliminated.

2. After selection of the slides, 2 cm parafilm squares are prepared with 1-1/2 cm holes perforated in the center. The parafilm chambers are then heat fixed to the slides.

For standardization of the technique, a 1×10^{-4} M solution of fluorescein was prepared as previously described and serial dilutions were made to 1×10^{-8} M. The pH was maintained at pH 7.0 in all dilutions with 0.01 M phosphate buffer.

Three drops of silicone oil (GE-SF-90) were placed in the chamber. A fine aerosol of the fluorescein was created and the drops were allowed to settle on the slide. The chamber was immediately sealed with a cover slip and the slides were placed in the dark. A 45-minute to 1-hour settling time is required for the drops to reach the bottom of the chamber. This time can be speeded up by low-speed centrifugation (See section 2.5.3).

2.4.3 Microscopic Measurement

The slides are examined on the modified Zeiss microscope. Its basic components are as follows:

The slide is illuminated with a 6-volt tungsten lamp. Current for the lamp is supplied by a heavy-duty storage battery, thus eliminating variation in readings caused by fluctuations in line voltage.

Exciting wavelengths are achieved through the use of a BG-12 filter placed directly under the dark field condensor. The condensor is adjusted so that it makes direct contact with the slide through a thin film of immersion oil. The 40X objective is then focused on the drops which have settled to the bottom of the slide. All focusing is performed under green light (540μ) to prevent degradation of the fluorescein.

When in focus, the drops will appear as two images as a result of the beam splitter. This device permits rapid scanning of the field for the selection of the proper drop size. Using the calibrated reticular, it was determined that when the edges of drops coincided they were 16 microns in diameter. All drops larger than this appeared as two overlapping spheres, and all drops smaller appeared as two distinct drops with varying distances between their edges. The beam splitter allows approximately 10% of the light to pass through the eyepieces and the remaining 90% to pass through an interference filter, which excludes all light below 500μ, and then into the Aminco Bowman photomultiplier (RCA 1P21). Fluorescent intensities are read in μ amperes.

2.4.4 Standard Curves for Fluorescein

Five dilutions of fluorescein were sprayed on the chambers. For this experiment, only 16-micron drops were measured. The results and deviations are shown in figure 6. Fluorescent intensity is linear, but not proportional to fluorescein concentration from 1×10^{-6} M to 5×10^{-5} M. A detailed explanation of this phenomenon is discussed in section 3. A second experiment was run to determine the effect of drop volume on fluorescent intensity with a given concentration of fluorescein. Droplets of 9-, 10-, 12-, 16-, and 19-micron diameters were measured. The results are shown in figure 7. Ten droplets of each diameter were measured. Lateral dispacements of the points for each dropsize indicate similar values. In this experiment, the fluorescein concentration was 5×10^{-6} M. The procedure for measurement was as previously described.

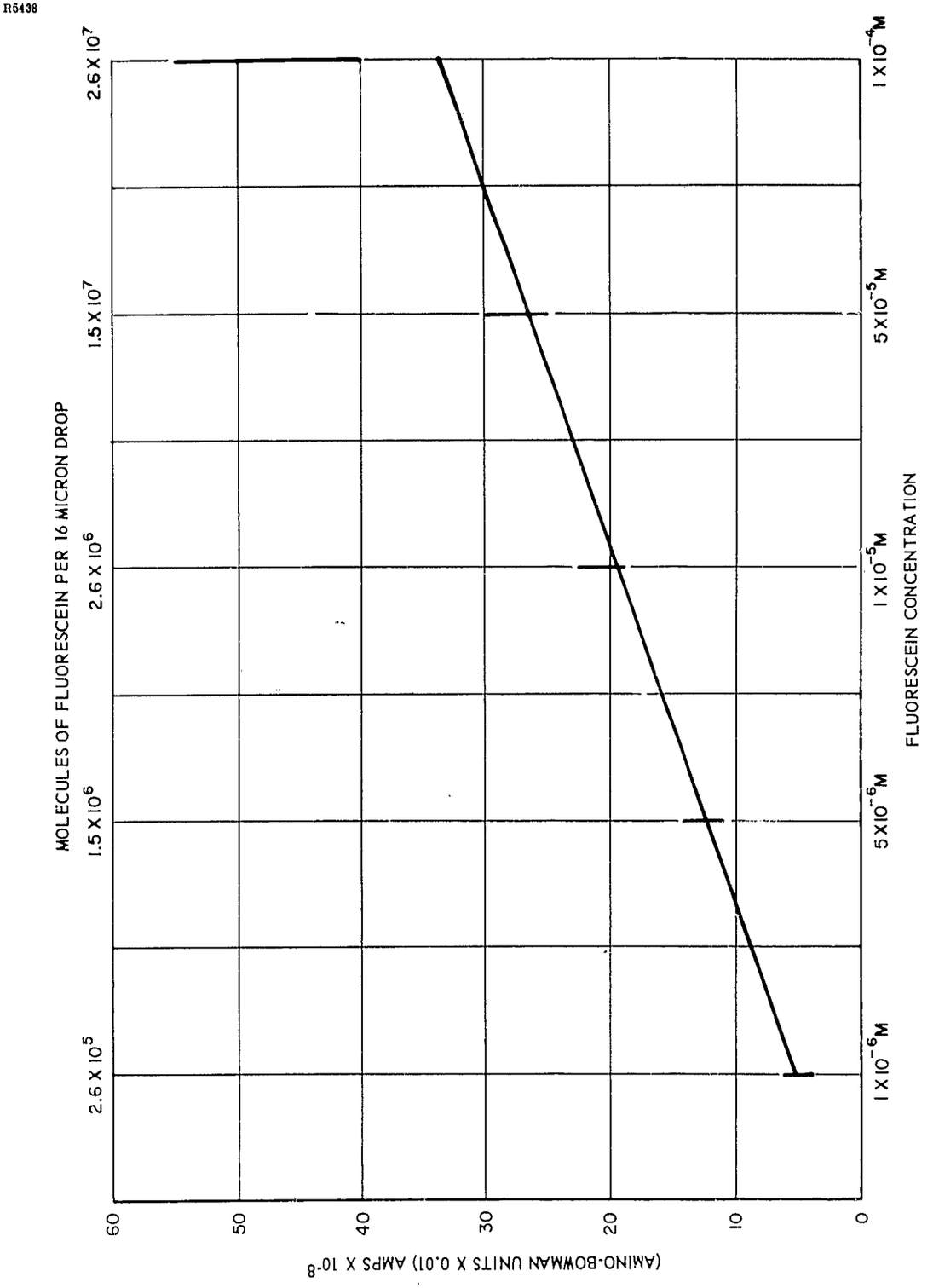

Figure 6. Fluorescent Intensity vs. Fluorescein Concentrations

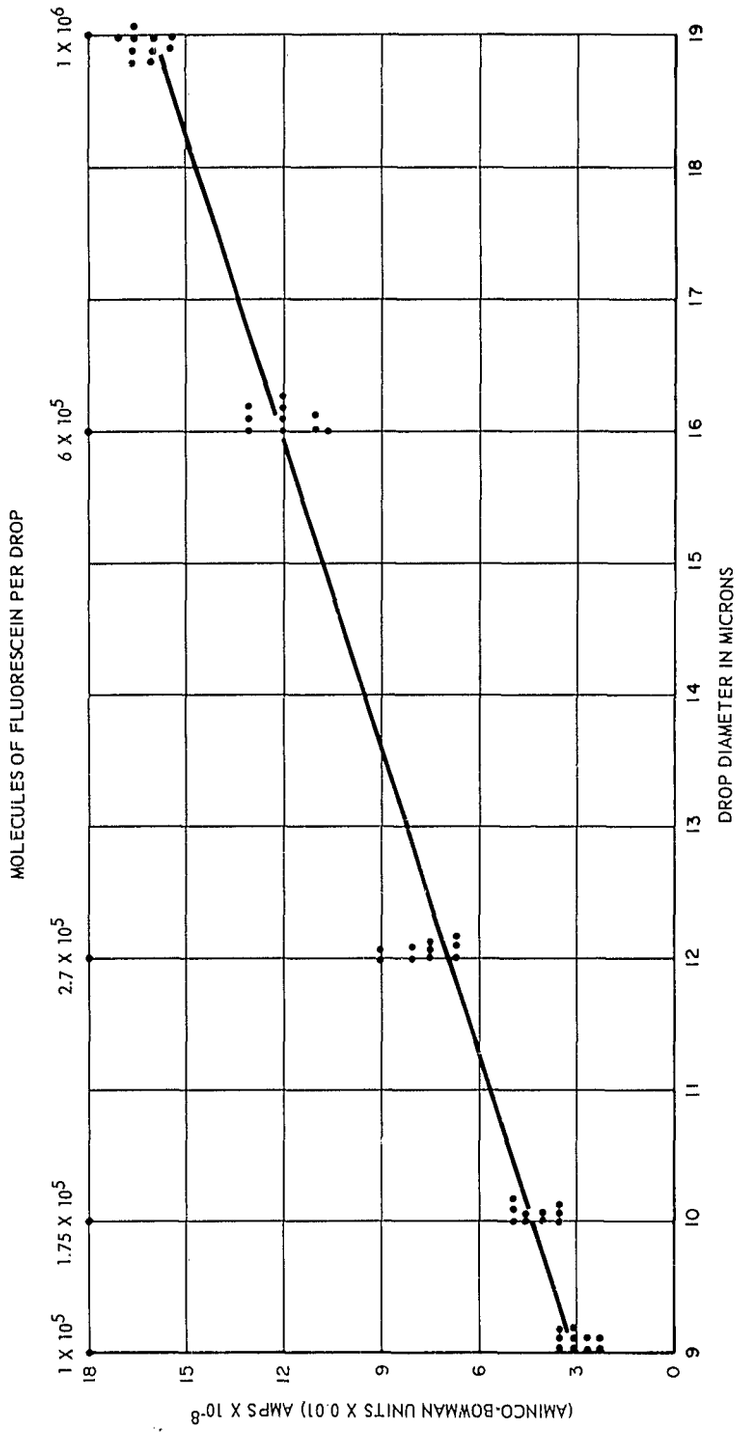

Figure 7. Fluorescent Intensities vs. Droplet Diameters

2.5 Measurement of Esterase Activity on the Microscope

2.5.1 Theoretical Analysis of Detection Sensitivity

Calculation of turnover numbers for bacteria tested were made with FDA as substrate. FMA was considered as the product of hydrolysis and all calculations were made on this basis. Because the exact product or combination of products of hydrolysis is unknown, the turnover numbers are only approximate figures and could vary ±25%. The figures represent the number of molecules of FDA hydrolyzed per minute per organism (table 1).

Table 1

TURNOVER NUMBERS FOR FDA ESTERASE OF BACTERIA*

	Turnover Number/Bacteria
Proteus vulgaris	4×10^4
Pseudomonas stutzeri	8×10^5
Saccharomyces cerevisiae	1.1×10^5
Shigella flexneri	5.4×10^4
Staphylococcus aureus	4.0×10^4
Serratia marcescens	7.0×10^4
Serratia marcescens (lyophilized)	4.5×10^3

*These values were calculated from data shown in figure 1.

The Aminco Bowman units were converted to μ moles of fluorescein monoacetate from figure 4, 2nd Quarterly Progress Report.

Even if one considers the deviation, it appears that a figure of 10^4 would be fairly representative for the bacterial esterase.

From the standard curve, figure 6, it can be seen that 2.6×10^5 molecules of fluorescein was readily detectable. Accordingly, assuming the turnover number to be 1×10^4, then in 20 minutes, one organism should produce detectable amounts of fluorescein, or 20 organisms could be detected in less than one minute.

2.5.2 Detection of Broken and Whole Bacterial Cells

To test the hypothesis above, the enzyme reaction was run on the microslides, using the same technique used for the standardization with fluorescein.

A suspension of lyophilized whole cells contained 8.2×10^{11} cells/ml. A cell-free preparation was made with the Nossal disintegrator by the same procedure as previously described (2nd Quarterly Progress Report). Ten-fold serial dilutions were made with the cell free-preparation. The following experiment was run, and the results are shown in figure 8.

No. of whole cells in cell free	1	2	3	4	5
8.2×10^{11}		0.3			
8.2×10^{10}			0.3		
8.2×10^{9}				0.3	
8.2×10^{8}					0.3
1×10^{-4} M FDA	0.5	0.5	0.5	0.5	0.5
0.5 M PO_4 pH 7.0	0.2	0.2	0.2	0.2	0.2
H_2O	0.3				

After spraying, the slides were incubated for 1 hour at 27°. A detectable amount of fluorescein was produced by less than one organism during the one-hour incubation.

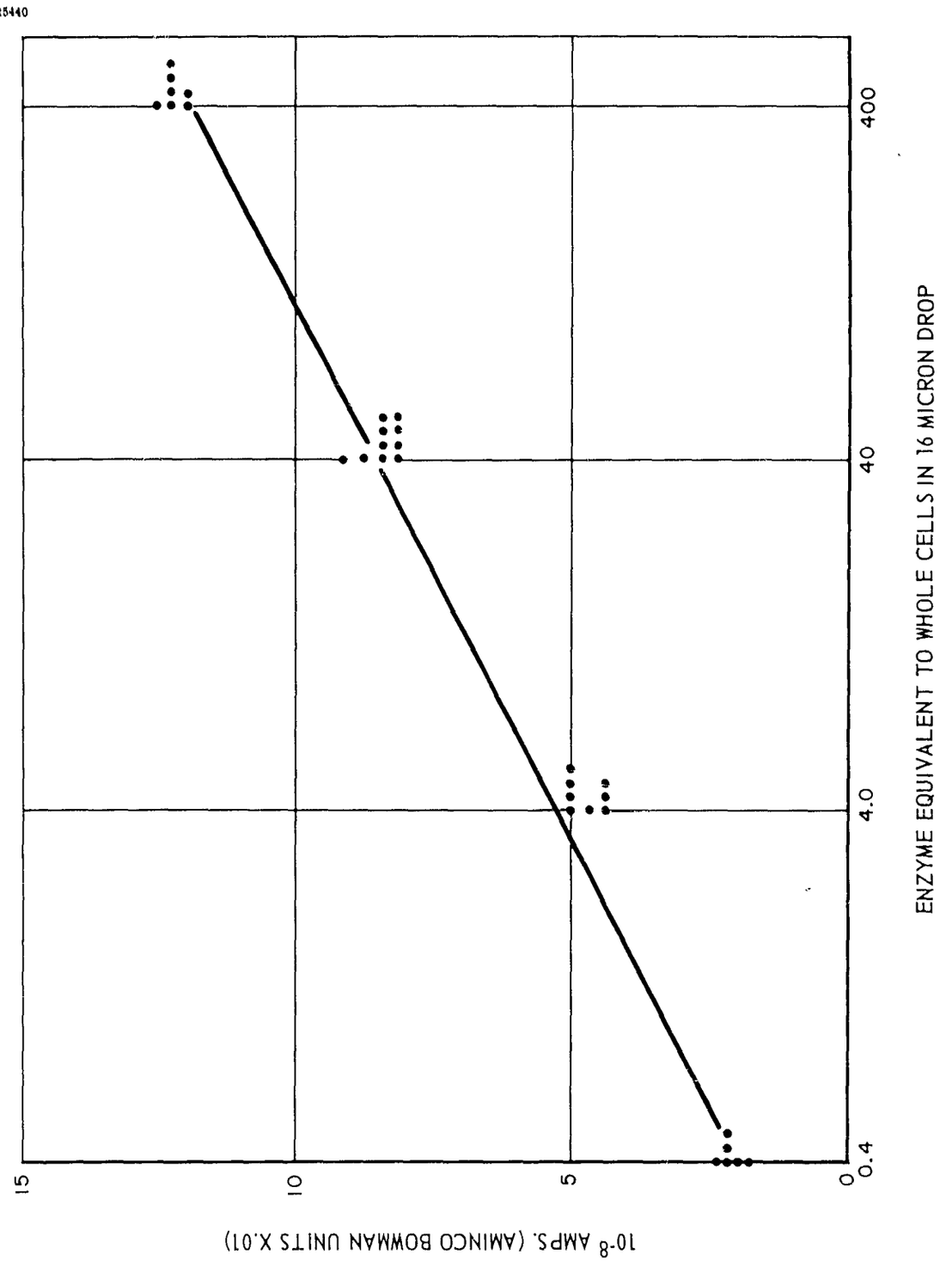

Figure 8. FDA Esterase (Cell Free) vs. Fluorescent Intensity

Figure 8 shows the linear response to enzyme concentration and establishes this technique as an ultrasensitive technique for the determination of small numbers of bacteria.

An experiment was run with whole cells by a procedure previously described for the cell-free study. All values were lower by approximately 50% and, for similar size drops, the results were quite variable. Presumably, this was caused by the random distribution of the bacterial cells in the drops. Attempts were made to count the cells in the individual droplets and, although it was evident that cells were present, no quantitation was possible.

2.5.3 Centrifugation to Decrease Incubation Time

Having decided to use only cell-free supernatants as the source of esterase enzyme, another experiment employing optimum conditions was run. The slides were incubated for 5 minutes at 35° in 0.2 M phosphate buffer pH 7.8 and centrifuged by the following method. The slides were taped to the large swinging buckets of the PR-2 centrifuge and spun for 60 seconds at 60 rpm. The slides were examined after a total incubation time of ten minutes. The values (figure 9) were almost identical to those obtained in figure 8, where incubation time was 1 hour, temperature was 27°, and pH was 7.0. Thus, it appears that, in 10 minutes, less than one organism can be detected by this method.

2.6 Differentiation of Fluorescein Derivatives (FDA, FMA, F)

As previously mentioned, part of the difficulty in quanitating the bacterial esterase, with FDA as the substrate, has been the determination of (1) the degree of purity of the esters, and (2) the product of the hydrolysis.

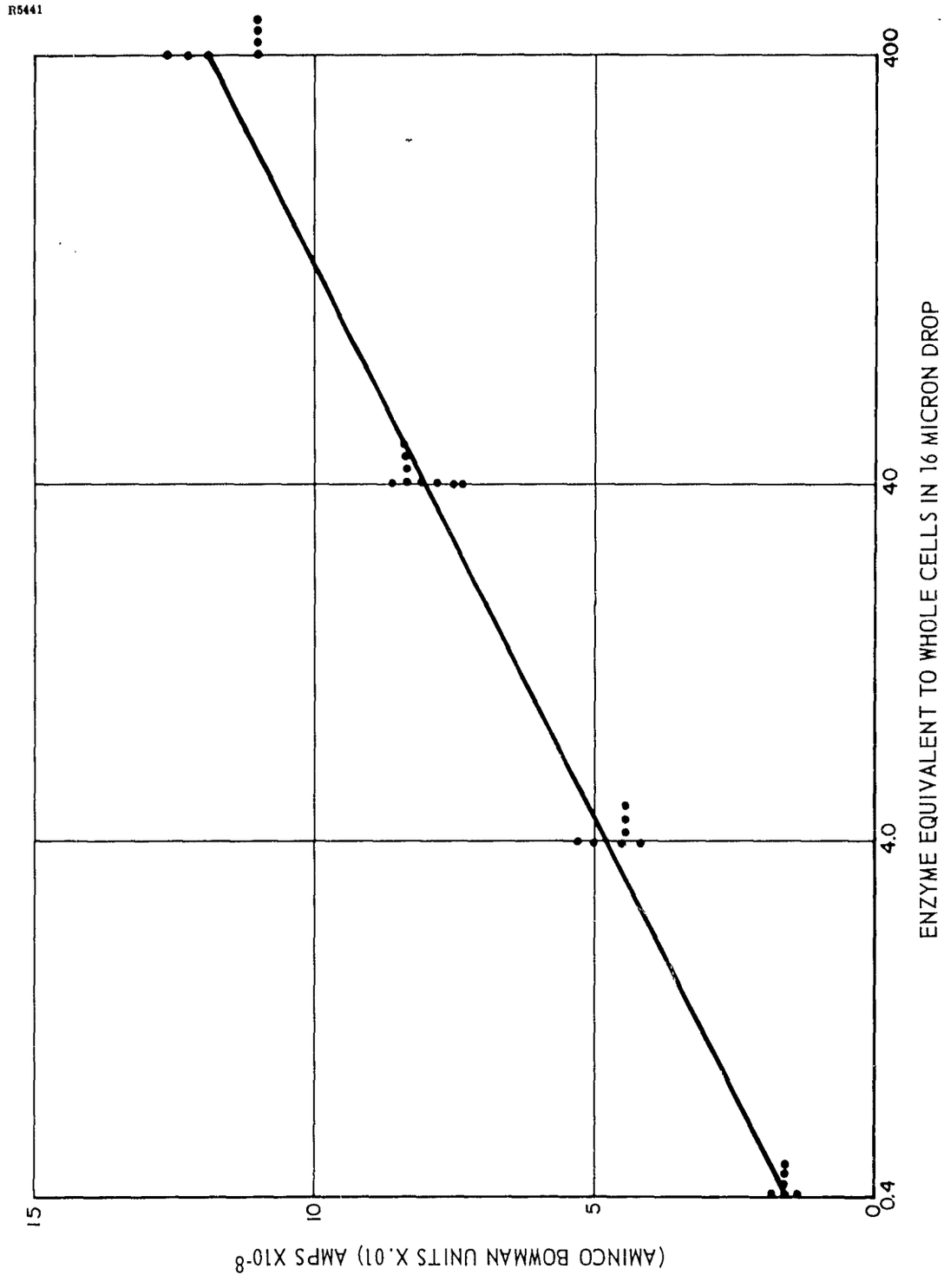

Figure 9. FDA Esterase (Cell Free) vs. Fluorescent Intensity

The first problem, the degree of purity, was approached along two lines: (1) infrared spectrum determination and (2) chromatographic techniques, paper and column.

2.6.1 Infrared Spectra

Two preparations of FDA and one preparation of FMA were synthesized in Melpar's laboratories. Infrared spectra of these esters and two samples of free base of fluorescein are shown in figures 10 through 13. The results of the IR spectra were as follows: A very strong hydroxyl peak at 2.9 μ was evident in the fluorescein and a lesser peak at 2.5 μ in the FMA. The 2.9 μ peak was absent in the FDA sample. In the unesterified free base, the hydroxyl group was quite strong and, in the partially esterified FMA, it was present but not as strong. Because of the absence of any peak in the FDA at 2.9 μ, the three compounds were thought to be relatively pure, containing only trace quantities of each other.

Secondly, in the free fluorescein, there was no acetate peak at 8.3 μ, where, in the FDA, this was a strong peak, and in the FMA, a much lesser peak.

In conclusion, it appeared that in the FDA preparation, the hydroxyl groups were bound and that only a small amount of fluorescein was contaminating the preparation. The reagent-grade fluorescein used in the synthesis compared favorably with the fluorescein standards used above. The question which remained was the purity of FMA. Was it a monoester or a mixture of FDA and F? To attempt to answer this question, both paper and column chromatography techniques were employed.

2.6.2 Paper Chromatography

Based on the preliminary results with IR experiments were designed to test chromatographically the purity of the fluorescein compound.

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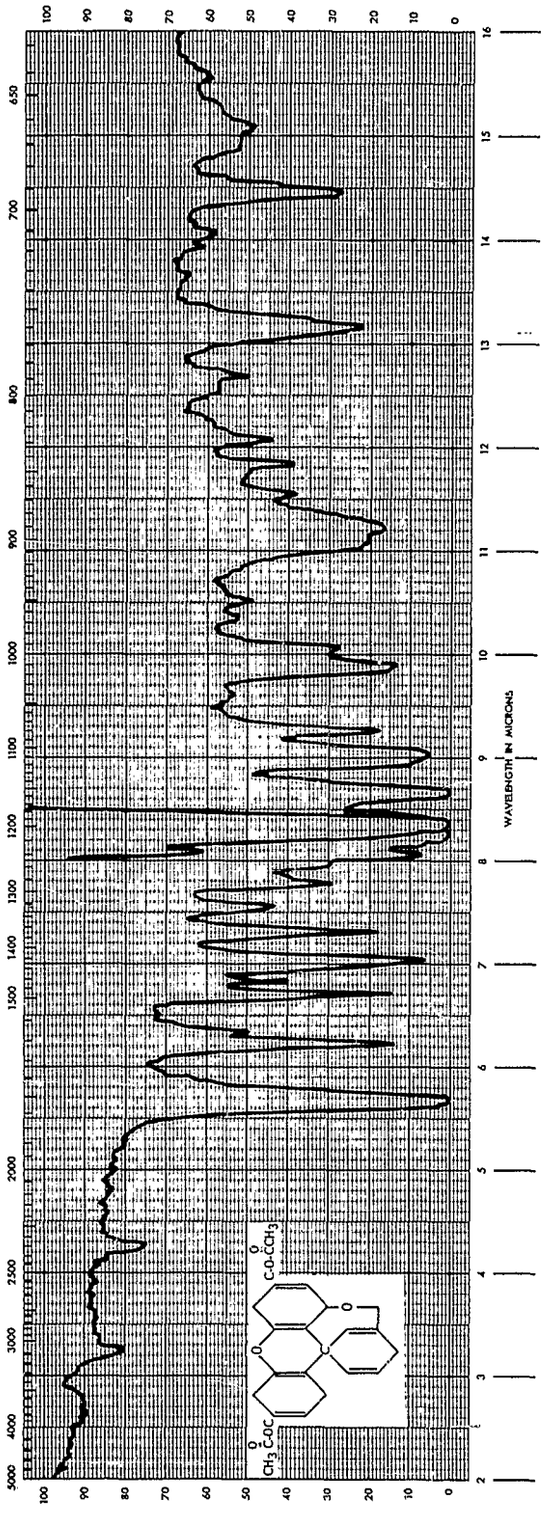

Figure 10. IR Spectra- Fluorescein Diacetate - Melpar No. 1

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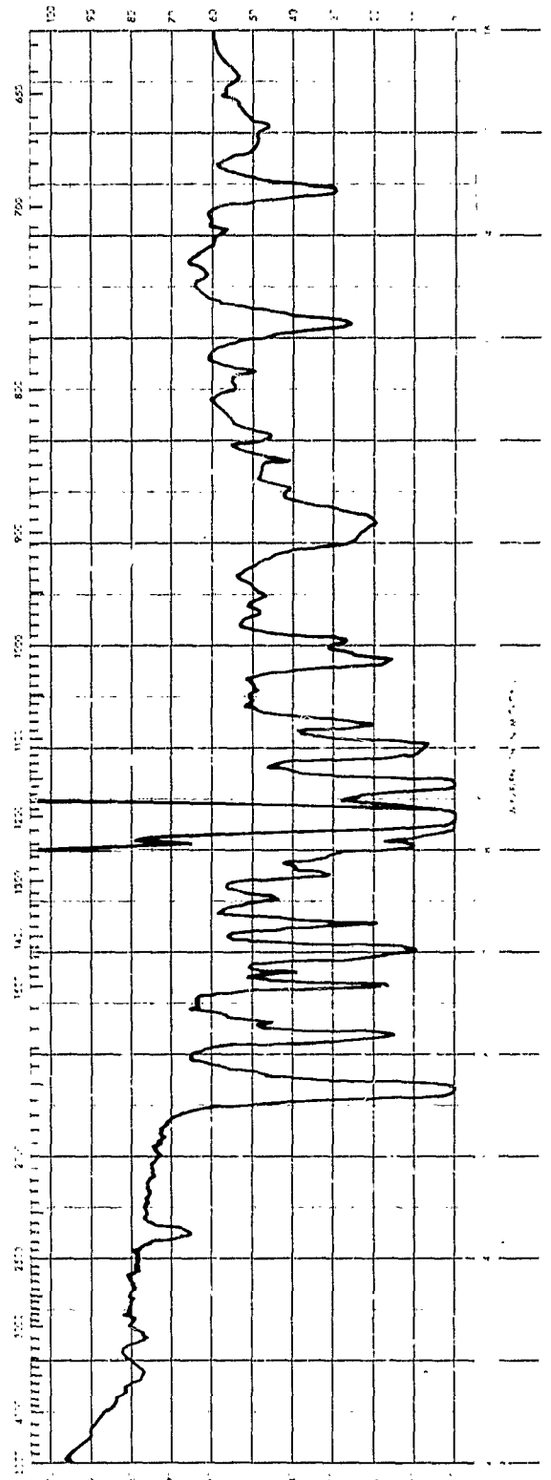

Figure 11. IR Spectra- Fluorescein Diacetate - Melpar No.2

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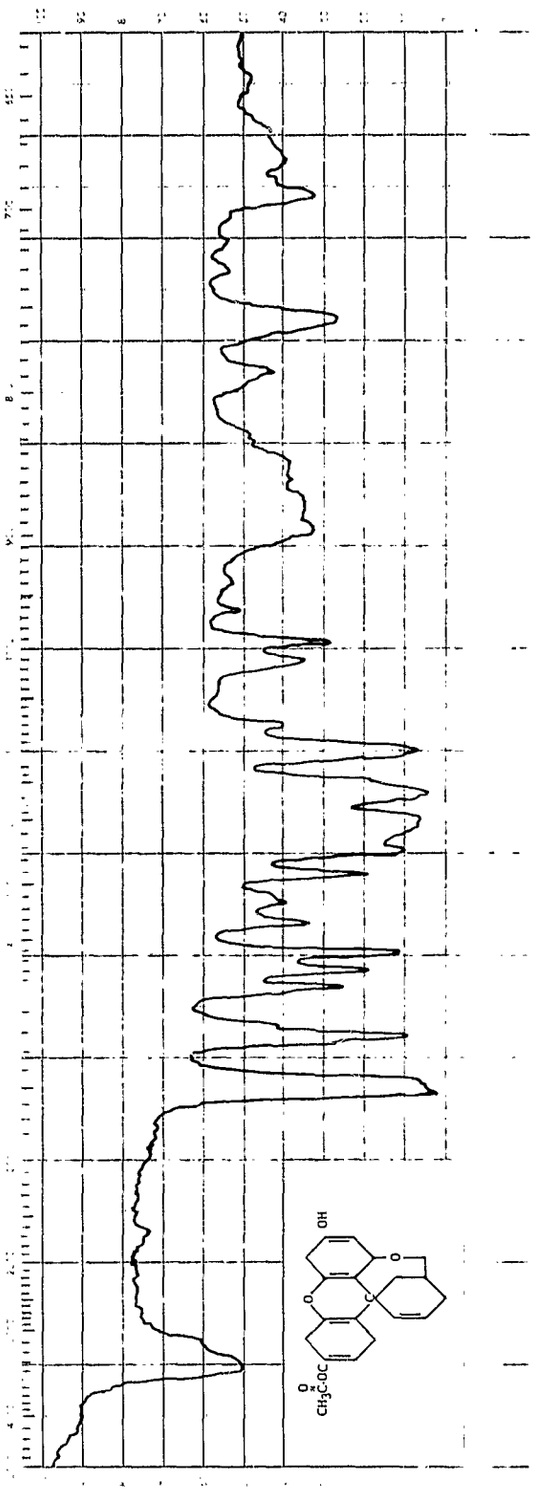

Figure 12. IR Spectra- Fluorescein Monoacetate

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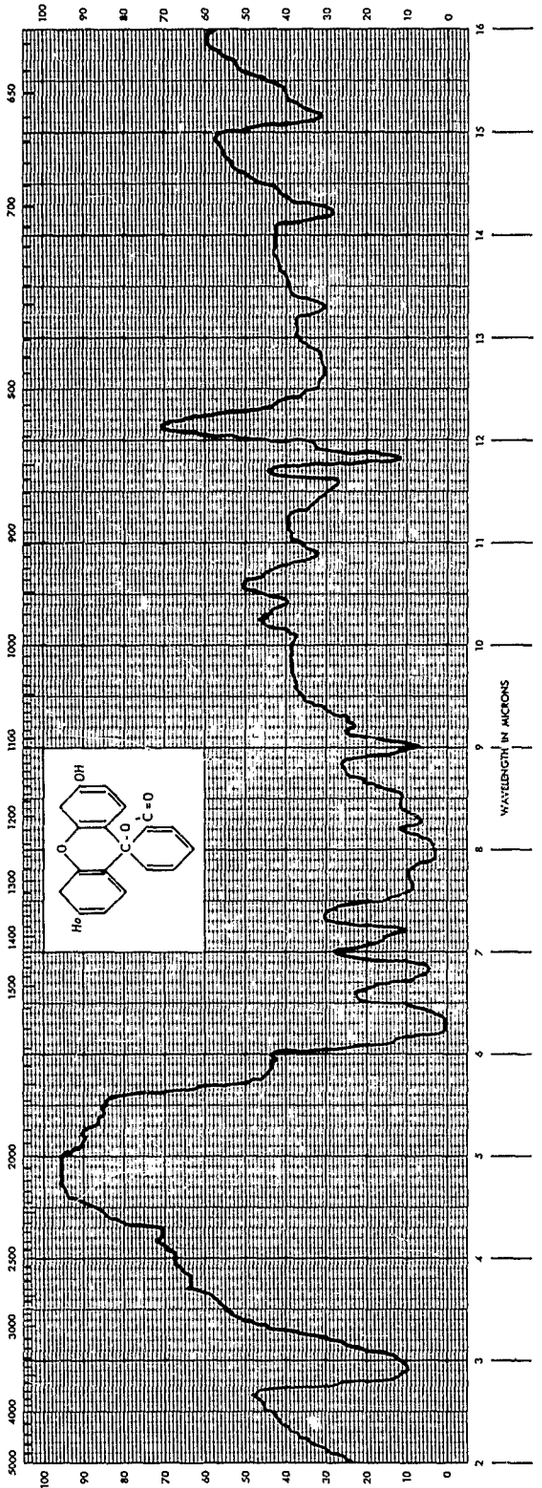


Figure 13. IR Spectra- Fluorescein

Five compounds were used in this study: FDA, FMA, fluorescein dibutyrate (FDB), and free fluorescein. The solvent system[11] 1:1:1 5% phenol, 3% sodium chloride, 1% ammonium hydroxide was used in the separation. Because of the low solubility of the compounds, acetone had to be used as the solvent. Ten µl samples, 1 mg per ml in 100 acetone, were spotted four inches from the end of a 22-inch strip of Whatman No. 1 filter paper. Duplicates were run in each case. The strips were dried and placed in the solvent system. After four hours, the front had moved to within one inch of the end of the strip. The strips were removed and examined under UV light (360 mµ). Bright spots with the fluorescein and FMA were detectable, with identical rf value. The FDA trailed the length of the paper and showed a weak fluorescence in a spot with similar characteristics to fluorescein. In an attempt to hydrolyze the remaining ester, the strips were treated with concentrated ammonium hydroxide. No further hydrolysis could be observed. Very little information was gained from this study which would help differentiate the various compounds. From a calculation of the rf values, it would appear that the FMA and fluorescein preparation behave identically in the solvent system used. FDA shows a similar rf value, and the long trailing would indicate that spontaneous hydrolysis is occurring throughout the separation, making identification of the compound impossible.

2.6.3 Column Chromatography

A second approach to the problem was with column chromatography, using Dowex 50W-X2 hydrogen form cation exchange resin. Cortis-Jones[4] successfully separated a mixture of 1-naphthol and resorcinol on a column 1.4 cm x 140 cm, using

acetone as the solvent. The procedure for separating the compound was as follows: A column 15 mm x150 mm was packed with the resin which had been equilibrated in acetone overnight. Two hundred mg of the FMA was dissolved in 5.0 ml of acetone and placed on the column. Fifty five 5.0 ml samples removed from the column were assayed for fluorescence in the Aminco-Bowman. The results indicated that three distinct fractions came off. The first was highly fluorescent and was collected in 2 tubes and was judged to be fluorescein; there was a break of 20 ml, and a second fraction came off, which was yellow in color, but when treated with sodium carbonate, was highly fluorescent. This fraction, presumably FMA, consisted of 30 tubes. Again, there was a break of 20 ml and a third fraction, colorless, came off. This fraction showed no fluorescence, but when treated with sodium carbonate, showed the characteristic bright green fluorescence. The total volume of this fraction was 25 ml. This third and final fraction was judged to be FDA. Although no quantitation was attempted, it did appear that this was a promising method for the separation of mixtures of similar compounds and should be further investigated.

2.7 B-galactosidase

One additional enzyme system which was under consideration as a possible detection system in this quarter was B-galactosidase.

Fluorescein di-galactoside was synthesized in Melpar's laboratories (see below) and was tested as a possible substrate with cell-free preparations of E. coli, K-12. These cells were grown on basal media and induced with lactate.[5] Results showed that the substrate was highly stable, and was readily hydrolyzed by these preparations of B-galactosidase.

The preparation of the enzyme was as follows: Cells in the early log phase were inoculated into a basal medium which was 1.7×10^{-4} M in lactose. The cells were agitated very rapidly at 37° for 90 minutes and then centrifuged for 45 minutes at 1000 x G in the cold. To the 2.0 ml cell suspension was added 0.4 ml reagent-grade toluene. The solution was then shaken for 5 minutes at 37°. The cells were allowed to settle and the toluene was extracted. The toluene treated cells were assayed with the fluorescein di-galactoside (FDG) 1×10^{-4} M and 0.5 M phosphate buffer pH 7.5. In 15 minutes, a considerable quantity of fluorescein was produced. No attempt was made to quantitate the results, as only a Yes or No answer was being sought. Extracts of S. marcescens and P. vulgaris were then assayed with the FDG. Results were negative in over 24 hours incubation with both organisms. Because the enzyme was not universally present, further work with this enzyme and substrate was considered inadvisable.

Synthesis of Fluorescein B-Galactoside[6]

Fluorescein digalactoside was prepared from 5 g of fluorescein purified through fluorescein diacetate. Commercial fluorescein (Eastman, acid form) was acetylated with acetic anhydride in pyridine. The reaction mixture was poured into cold water from which the acetate was filtered off. The product was recrystallized three times from ethyl acetate. Fluorescein was obtained from its acetate by dissolving it in alcoholic KOH 1N and boiling for one-half hour over steam bath. The liquid was driven off completely in a flash evaporator at room temperature; the syrup was dissolved in dilute NaOH and fluorescein was precipitated with 1N HCl, filtered and dried.

The galactoside was made from 5 g of fluorescein mixed with 12.4 g of brom-tetraacetyl galactose, 3.5 g Ag_2O (freshly made) in 7.5 ml of dried benzene (dried over sodium sulphate). Four drops of freshly distilled quinoline were added and the mixture was stirred with a magnetic stirrer in the dark at room temperature for three days. After this period, the suspension was filtered and the benzene solution was washed with water, dilute NaOH, and again with water. Then it was dried overnight over sodium sulphate. This solution contains the digalactoside and monogalactoside. The pure compounds were separated on a 100 g silicic acid column, using a linear gradient of 700 ml benzene and 700 ml benzene/ethyl acetate = 20:80. The digalactoside (about 2.3 g) comes off first and is recognized by the fluorescence given after boiling with 1N alcoholic KOH. The monogalactoside comes off with pure ehtyl acetate. Both compounds were obtained after evaporating the solvent and crystallization from methanol. Deacetylation of the final product was done as usual with sodium methylate at 0° for one to two hours. The deacetylated digalactoside was purified by crystallization from acetone.

2.8 Papain

Work on the papain system continued with very satisfactory results. As previously reported, crystalline papain was prepared by the method of Kimmel and Smith.[7] All colorimetric assays were performed on the B and L Spectronic 20 or on the Klett Summerson colorimeter. Mercuri-papain was obtained from Sigma Chemical Co., St. Louis, Mo.

2.8.1 Standard Curve for p-nitrophenol

A standard curve with p-nitrophenol was run on the Spectronic 20 as follows: Serial dilution of 1×10^{-4} Molar

stock solution of p-nitrophenol was made in 0.5 M phosphate buffer pH 7.0. Ten ml of each dilution was measured colorimetrically at 420 mμ. The results are shown in figure 14.

2.8.2 Activation of Crystalline Papain by Cysteine

If one assumes that, to activate one mole of papain, it requires one mole of reducing agent, a straight-line relationship should exist between substrate hydrolyzed and reducing agent utilized.

In the following activation study, only a small portion of the papain present is being activated, and an increase in cysteine concentration should show an increase in activity, provided that substrate concentration is not limiting.

As figure 15 shows, at cysteine concentrations of 5×10^{-4}, 2.5×10^{-4}, and 1×10^{-4} molar, the substrate concentration after 1.5 minutes is limiting. At 5×10^{-5} M, straight-line relationship exists from 0 to 65 m moles of CTN hydrolyzed. The protocol for the experiment is as follows:

	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml
Papain 0.1 mg/ml	1.0	1.0	1.0	1.0	1.0	1.0
CTN 2×10^{-4} M cysteine (neutralized)	1.0	1.0	1.0	1.0	1.0	1.0
5×10^{-4} M	0.5					
2.5×10^{-4} M		0.5				
1×10^{-4} M			0.5			
5×10^{-5} M				0.5		
1×10^{-5} M					0.5	
EDTA 1×10^{-2} M	0.3	0.3	0.3	0.3	0.3	0.3
.5 M PO_4 = pH 7.0	0.2	0.2	0.2	0.2	0.2	0.2
H_2O						0.5

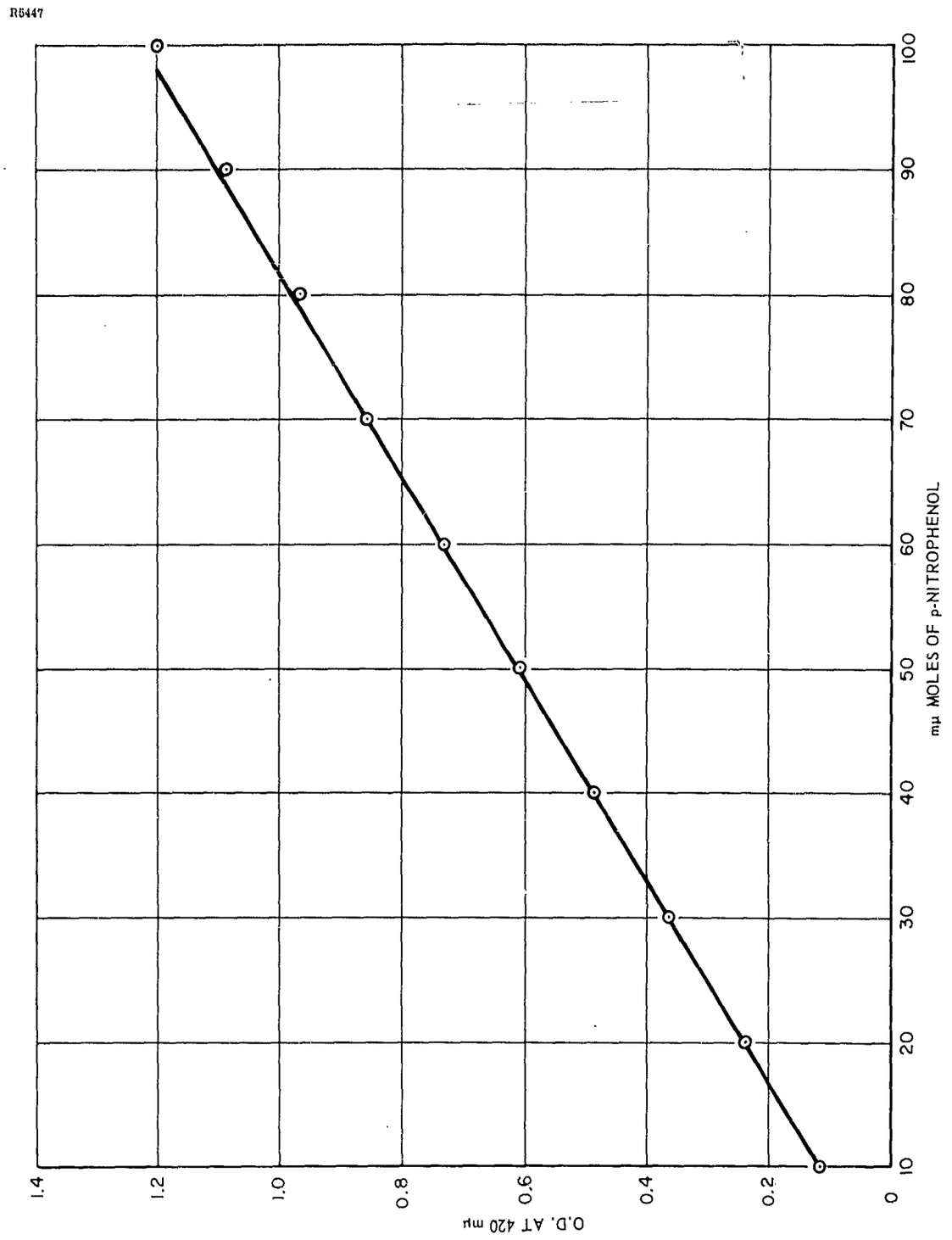


Figure 14. p-nitrophenol Standard Curve

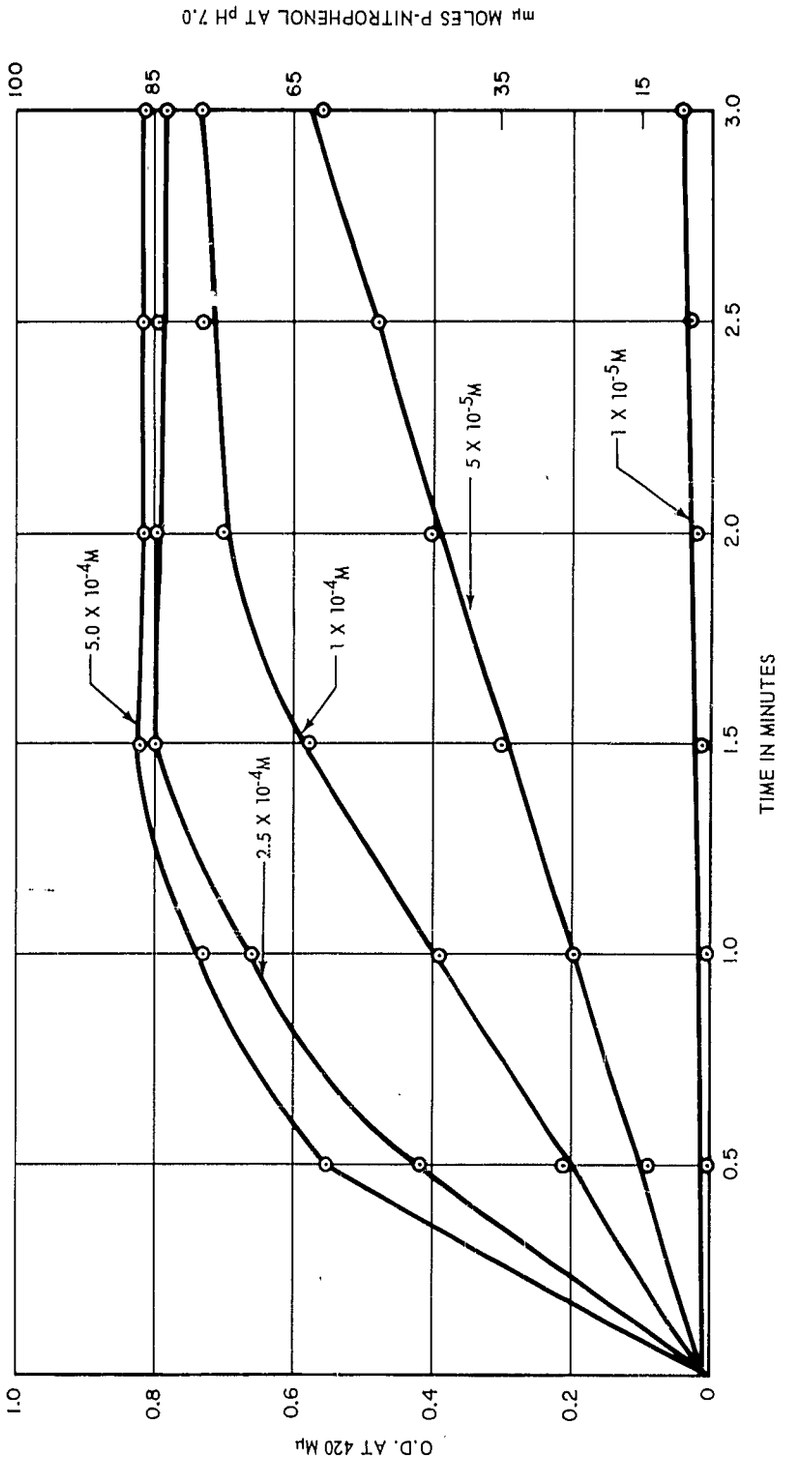


Figure 15. Papain Activation vs. Cysteine Concentration

The reaction mixture was incubated at 27°C and the p-nitrophenol liberated was measured at 30-second intervals.

2.8.3 Activation of Mercuri-Papain by Cysteine

The effect of varying cysteine concentration on the activation of mercuri-papain (obtained from Sigma Chemical Co.) was studied. The procedure was the same as that used in the H_2O_2 inactivated preparation. The mercuri-papain was a very active preparation with substrate becoming limiting with 1×10^{-4} and 5×10^{-4} M cysteine in 0.5 minutes (figure 16). There was no advantage in using the mercuri-papain other than its apparent higher activity. Therefore, all additional activation studies were performed with the H_2O_2 inactivated preparation.

2.8.4 Papain Activation by Bacteria

Having activated papain with cysteine, it was important to determine to what extent it might be activated by certain bacteria. Both whole cells and cell-free supernatants of Serratia marcescens were tested for their ability to activate papain. The following experiment was run, and the results are shown in figure 17.

	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	7 ml
Papain 0.1 mg/ml	2.0	2.0			2.0	2.0	
CTN 2×10^{-4} M	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Whole cells 5×10^{10}	1.0		1.0				
Cell free					1.0		1.0
EDTA 1×10^{-2} M	0.6	0.6	0.6	0.6	0.6	0.6	0.6
M PO_4 pH 7.0	0.4	0.4	0.4	0.4	0.4	0.4	0.4
H_2O	4.0	5.0	6.0	7.0	5.0	5.0	6.0

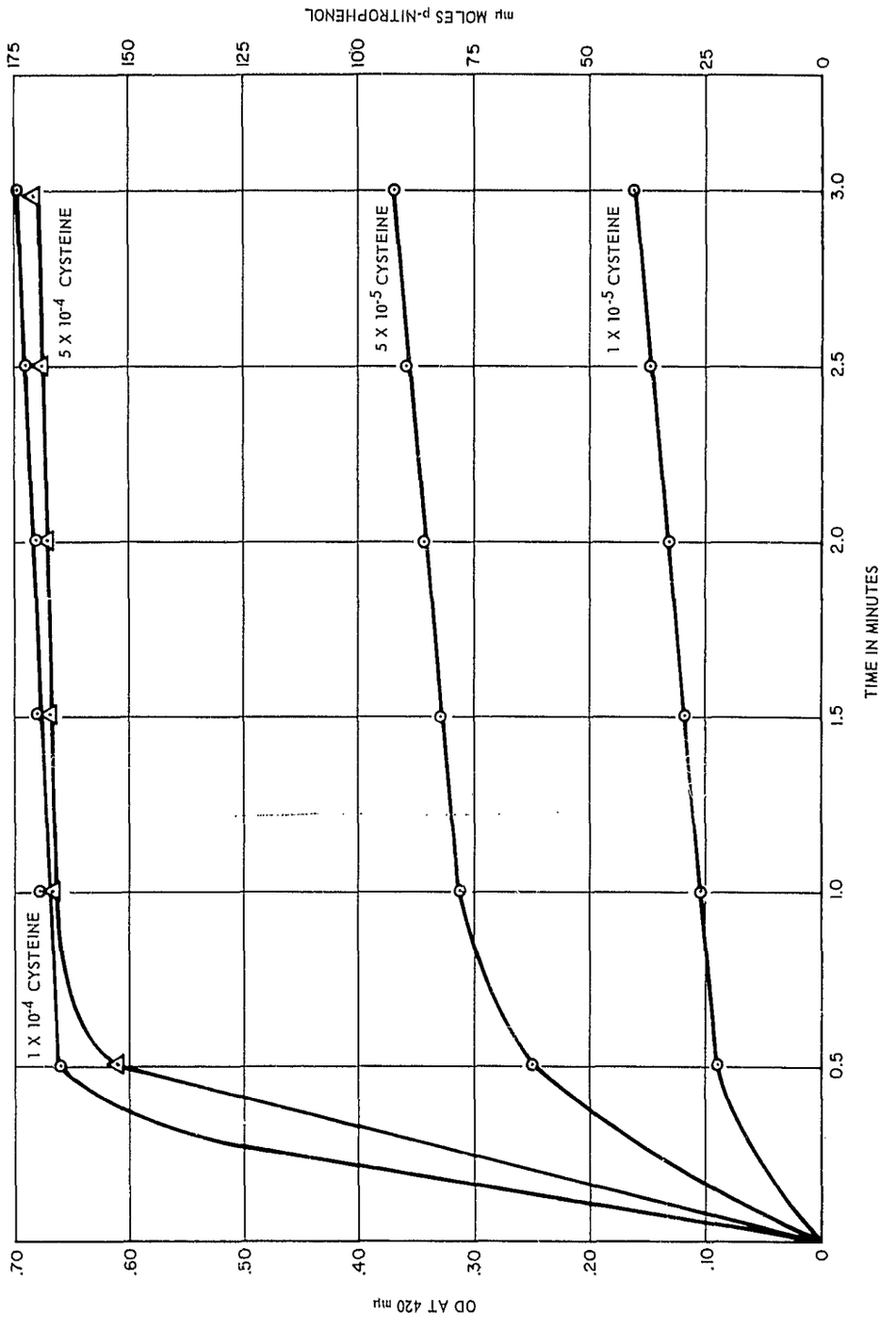


Figure 16. Mercuripapain Activation by Cysteine

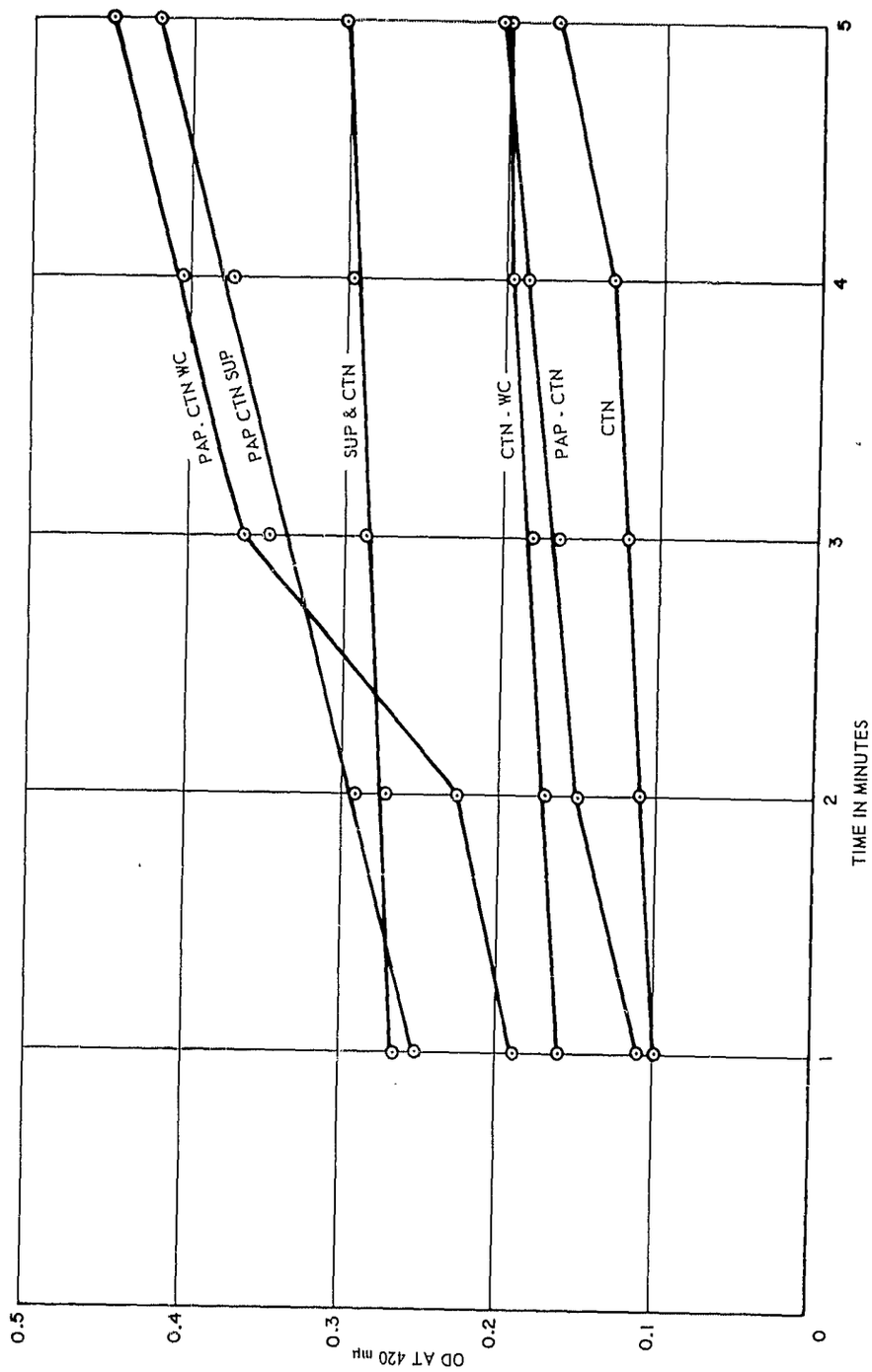
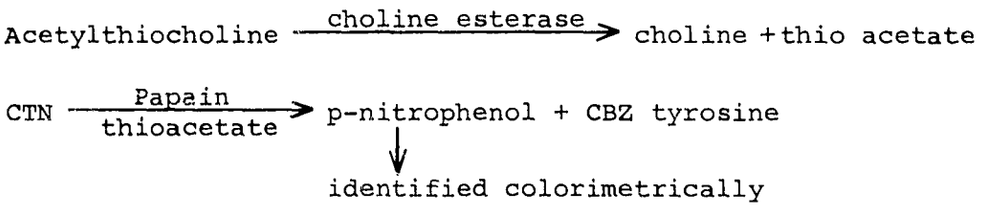


Figure 17. Papain Activation by Whole Cells and Cell Free of S. marcescens

Because a certain amount of activation was observed with S. marcescens, it was decided that the organisms previously screened for FDA esterase should be tested for possible papain activation. Six organisms were tested, using the identical procedure as for S. marcescens. The results are shown in figure 18. All organisms gave a small activation of papain, and small amounts of hydrolyzed CTN. If the bacterial cells themselves will activate papain, and do so in small enough numbers, this might be a feasible detection system. From this preliminary data, however, it appears that background levels would run so high that it would take far too many bacteria to give a positive result.

2.8.5 Papain Activation in Coupled Enzyme System

With the background problem in mind, the next approach was to demonstrate the feasibility of enzyme amplification by coupling two enzyme systems, as shown diagramatically below:



The protocol for the experiment is as follows:

	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	7 ml	8 ml
Papain 0.1 mg/ml	2.0		2.0	2.0		2.0		
Acetyl thiocholine iodide (ATC) 2×10^{-4} M	1.0	1.0		1.0			1.0	
Cholinesterase 1 mg/ml	1.0	1.0	1.0		1.0			
CTN 2×10^{-4} M	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
EDTA 1×10^{-2} M	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
.5 M PO_4 pH 7.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
H_2O	3.0	5.0	4.0	4.0	6.0	5.0	6.0	7.0

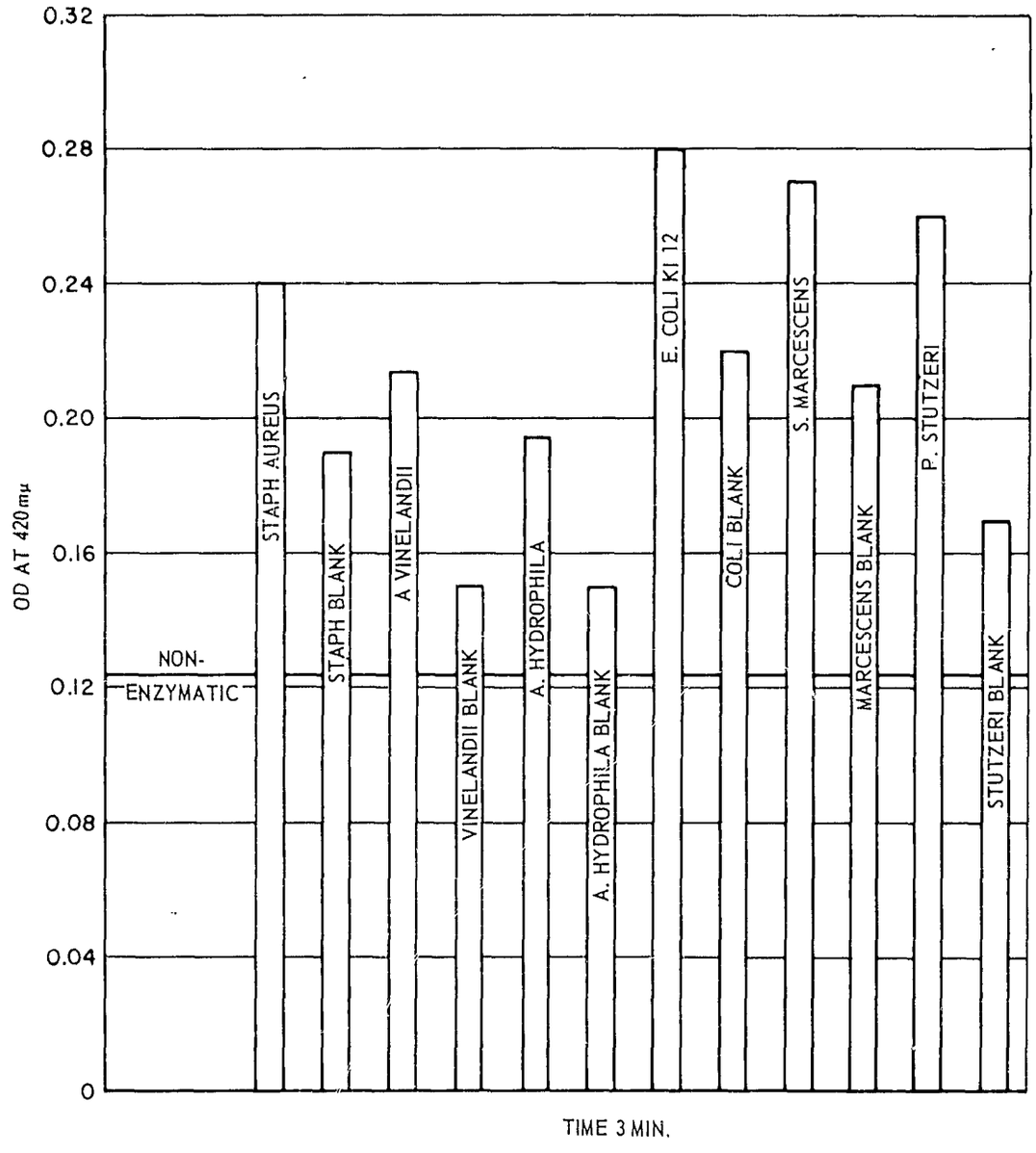


Figure 18. Papain Activation by Six Bacteria

These preliminary results (figure 19) show that the coupling system does work and that the system appears feasible. A search for thio compounds for substrates for the bacterial esterase should be made. Any thiol ester which, upon hydrolysis, will liberate a sulfhydryl will be suitable and the synthesis of such compounds, i.e., thiophenylacetate and thio ethyl acetate should be investigated. Similar experiments had previously been run in Melpar's laboratory with mercuri-papain. This experiment duplicated those results using H_2O_2 inactivated papain instead of mercuri-papain. From the data in figure 19, the coupled enzyme approach appears to be very promising.

2.9 Discussion of Results and Application of the Microscopic Technique

The results described in section 2.5.2 should be evaluated in terms of some theoretical considerations. From the data in figure 8, it can be seen that a value of 5 is equal to the fluorescent intensity produced by the hydrolysis of FDA by 4 organisms. The calculated turnover number of S. marcescens is 4.5×10^3 (table 1). Therefore, 4 organisms should produce approximately 1×10^6 molecules of FDA in 60 minutes. From the standard curve, a value of 5 is equal to the fluorescent intensity of 2.5×10^5 molecules of fluorescein. If one considers the possible errors in calculating the turnover number, the actual and the theoretical values are in very good agreement.

In section 2.5.2, activities with whole cells were significantly lower (about 1/2) than those with the cell-free supernatant, an observation that was anticipated. This data is in good agreement with that previously shown in figure 1 in the second quarterly report. However, it should be emphasized that,

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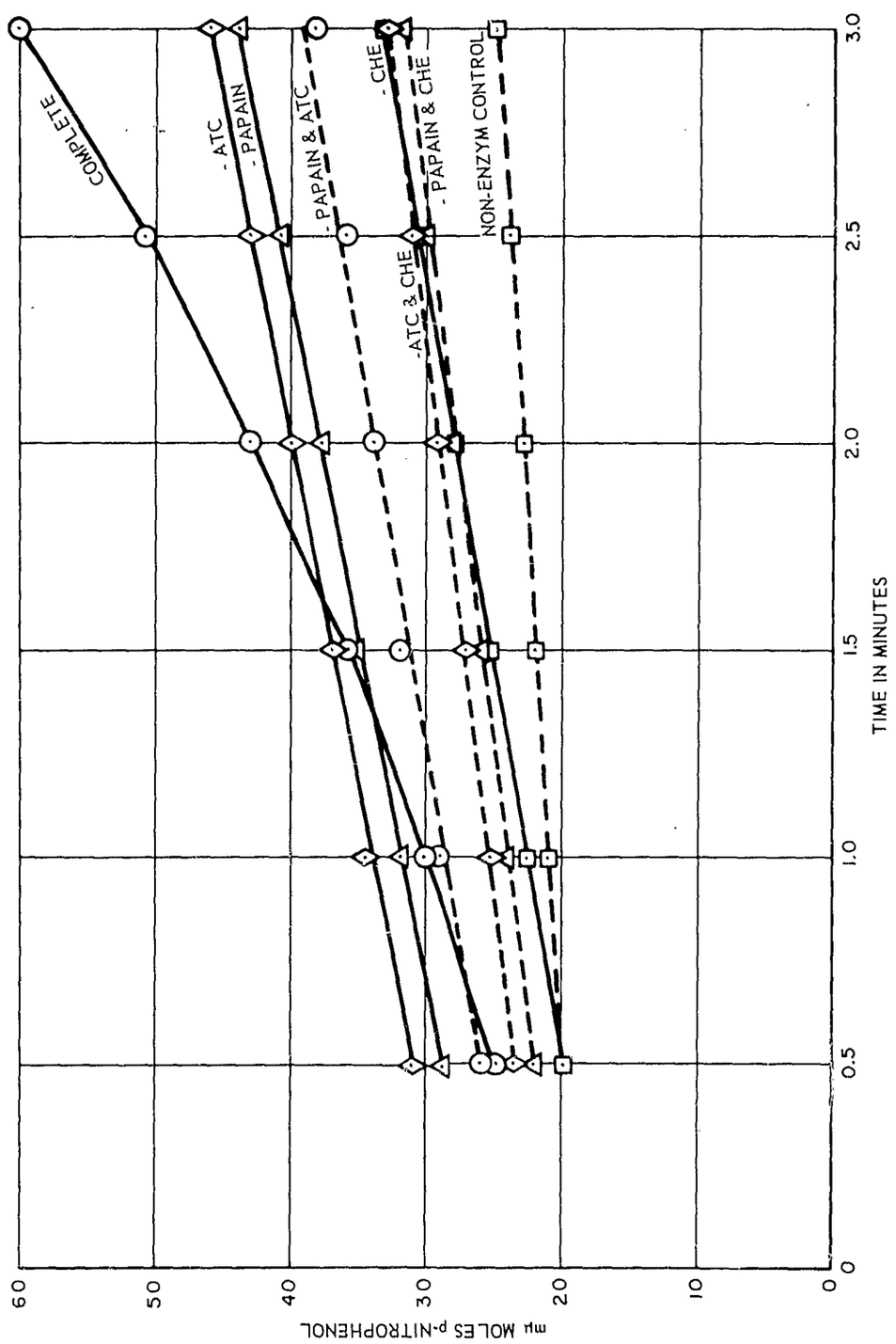

Figure 19. Coupling of Cholinesterase with Papain

for purposes of detection there would be little difference between one broken bacteria or two whole bacteria when one considers that the goal is to detect 1 to 100 bacteria. As expected, it is statistically more difficult to obtain good quantitation with bacteria in the microdroplets than with soluble cell-free enzyme. For this reason, most of the experiments were carried out with cell-free preparations.

It should be emphasized that the data obtained on the microscope is still rather crude, and it is possible that with greater refinement, i.e., defining the product of hydrolysis, and more exact calculations of turnover numbers, the method will be greatly improved.

The amplified enzyme approach is still under serious consideration. At present, only very rough data is available to evaluate its merits. It may be possible to increase the theoretical sensitivity by a factor of 100 if the p-nitrophenol ester of the papain substrate could be replaced by a fluorescent molecule.

Application of other enzyme systems to the microsopic technique is another important consideration. A system which would reduce DPN^+ (Diphosphopyridine nucleotide) and TPN^+ (triphosphopyridine nucleotide) to DPNH and TPNH respectively might be very good. DPNH is highly fluorescent with an absorption maxima at 340 mμ and an associated fluorescence emission in the 440-460 mμ region.[12] Both coenzymes are easily and reversibly reduced by various reducing agents and by specific dehydrogenases. These enzymes should be universally present in bacteria. The reverse oxidation of the reduced form is carried out by many dehydrogenases and certain

chemical agents. It is possible to measure 10^{-13} moles of either oxidized or reduced pyridine nucelotide in 10 μl volumes.[2] By using the appropriate accessory enzymes, practically any enzyme system can be made to produce an oxidation of DPNH or TPNH or a reduction of DPN^+ or TPN^+. Thus, if in a volume of 1×10^{-2} ml, 10^{10} molecules can be measured, then in a volume of 10^{-9} ml, which is the volume required for the microscope technique, it should be possible to detect 10^3 molecules of DPN^+ or TPN^+. Such a system can be directly applied to the microscope technique and may offer an increase in sensitivity over the present fluorescein substrates of as much as 100 times.

3. SUMMARY AND CONCLUSIONS

Three factors affecting FDA esterase were investigated. It was found that (1) salt inhibited the enzyme and did not quench fluorescence, (2) anaerobes have high levels of FDA esterase activity, and (3) the enzyme was considerably less active with fluorescein dibutyrate; thus, the conclusion that longer-chain esters would probably be poorer substrates. Partial and complete heat denaturation of the enzymes responsible for hydrolysis of FDA indicated that two enzymes might be present in the bacterial esterase system, one which hydrolyzes FDA to FMA, and one which hydrolyzes FMA to fluorescein.

Thirteen organisms representing several pathogenic genera were screened for FDA esterase activity. In all cases, high esterase activities were demonstrated and, in most cases, the activities were higher than that with S. marcescens.

Work on the microscopic technique was continued with very satisfactory results. Standard curves were run with fluorescein in the microdroplets, and the bacterial esterase was assayed in the microdroplets. In 10 minutes, one broken microorganism or two whole bacteria became detectable. This demonstrates that the enzyme approach for detection is rapid, sensitive and specific. Certain inadequacies were pointed out, namely, that the time required for the drops to settle should be greatly reduced. Further effort here will be required.

Failure to demonstrate a response linear to concentration with either fluorescein or the product of the hydrolysis for a given droplet volume can be attributed to the nature of the fluorescein molecule. Rotman[1] encountered the same difficulty

and observed a similar disproportion between concentration and fluorescent intensity. Concentration is a critical factor in measuring its fluorescence at a given pH. Self-adsorption occurs beyond a critical concentration. Bowen and Wolzes[10] state that, for a linear response, the solution must absorb less than 5% of the exciting radiation. They point out that, with increased light intensity, higher concentrations can be measured, provided one takes into consideration the rate of photodecomposition and light scattering under the various conditions. Therefore, it is only in the region where no significant absorption is occurring that fluorescence is proportional to concentration.

The need for the isolation of the product of the hydrolysis was established; without this knowledge, it may be difficult to give exact quantitation for all organisms.

Attempts were made to define more clearly the structure of FDA, FMA, and fluorescein. The IR spectra suggested that FDA did exist with only trace quantities of contaminating fluorescein. The existence of FMA, however, was still doubtful. Column chromatography of FMA showed three separate bands, indicating that FDA, FMA, and fluorescein may be present in the preparation of FMA.

The effort on the papain study was increased, and quantitation studies, with varying concentrations of activator, were made. Two forms of inactive papain were tested with cysteine. Mercuri-papain exhibited higher activities than the H_2O_2 inactivated preparations did. This, however, was not deemed too important as backgrounds were similar with both preparations. In addition to chemical activation, the effect of bacteria on papain activation was tested. It was found that all bacteria tested showed papain

activation as well as exhibiting some activity toward the CTN substrate. Both whole cells and cell-free supernatants were tested with no significant differences between the two. It appeared that it might be unnecessary to use a two-enzyme system for papain activation, but instead to activate with only the bacteria. Levels were not sufficiently high, however, to warrant further investigation.

The possibility of enzyme amplification by enzymatic coupling was tested using acetyl thiocholine-cholinesterase coupled with papain.

The results demonstrated the feasibility of such a system, and showed that it was an area where further effort was needed.

4. PROPOSED EFFORT FOR NEXT QUARTER

Work during the next quarter will continue in several different areas.

New substrates for application in the microscopic technique will be investigated. Presently, several naphthol esters are under consideration and will be tested. In the case of the α and B naphthol esters, the ester itself is fluorescent and, upon hydrolysis, shows a shift in emission and exciting spectra. By the selection of appropriate wavelengths, the free phenol can be easily distinguished from the ester.

Standard curves will be prepared to determine the sensitivity of our system with naphthyl-fluorescein, which may be superior in some aspects to the aliphatic fluorescein substitutions. This investigation will determine if the esters of these compounds should be studied.

In addition to the investigation of other substrates, further work will be done in an attempt to optimize further the conditions necessary for maximum esterase activity. This work will include the testing of activators and the limitation of any possible inhibitions. Further refinement of the microspectrophotometer, with the addition of a monochromater to supply the exciting wavelengths, should increase the versatility of the technique, allowing the use of numerous substrates.

The effort of papain activation and enzyme amplification will continue through the synthesis of new substrates. It appears that phenyl thioacetate and phenylthiobutyrate should be excellent possibilities, as substrates, for the esterase, papain system.

If time permits, a second group of substrates which should be tested are esters of fluorescent dyes which, upon hydrolysis,

would bind to the cell wall. Such compounds as "biofluor" disodium salt 4,4'-bis 4-anilino-6-bis (2-hydroxyethyl) amino-s-triazin-2-ylamino -2-2'-stilbene disulfonic acid will be considered for this purpose. Rees et al.[8] described techniques using n benzyl derivatives of 1-anilino naphthalene-8-sulfonic acid and 3-chloro-6-methoxy 9 aminoacridine. These two chemicals, in solution, are nonfluorescent but, when adsorbed to protein, are highly fluorescent. It may be possible to make nonfluorescent esters of these compounds and then, by hydrolysis, selectively stain the cell wall.

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