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# The Effect of Tunicamycin
# on
# Secretion of Acid Phosphatase
# in Yeast

By

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THESIS

for the

DEGREE OF BACHELOR OF SCIENCE

IN

CHEMICAL ENGINEERING

College of Liberal Arts and Sciences

University of Illinois

Urbana, Illinois

1990

## TABLE OF CONTENTS

## ABSTRACT

This report focuses on four experiments concerning the two strains of yeast (PYE and PYEαFPHO), their growth rates, and the rate at which they secrete acid phosphatase. One experiment tries to determine the optimal concentration of casamino acids to add to the growth medium. Another compares the results of two assays that determine the plasmid containing fraction of colonies. The next experiment measures the specific activities of the supernatant and the whole cells and then uses these values along with the Klett and time to determine the rate of production of acid phosphatase in the cells and in the growth medium. The last experiment explores the effect tunicamycin has on the growth rate of the yeast cells and secretion rate of acid phosphatase from these cells.

# INTRODUCTION

The biological aspects of chemical engineering are becoming very popular at Universities, in fact a faculty member was just added to the chemical engineering staff at the University of Illinois last fall, who specializes in biotechnology. I decided to do my senior research in this area because I spent the previous two years working for the microbiology department.

I have spent the last two and a half semesters working with yeast cells and determining the growth rate of the cells and the secretion rate of proteins from the yeast cells. This research is important because in recent years, scientists have been genetically programming yeast cells to produce foreign proteins and some day, the beneficial, end result of this process will be the mass production of medically important proteins that only occur in small quantities naturally. However, in order to commercially produce these proteins we need more knowledge of the pathway the protein takes as it is secreted from the yeast cell. We also need an optimal media for producing and purifying the protein. My research concentrates on optimizing the secretion of the protein s. pombe acid phosphatase in yeast.

My studies have focused on two strains of yeast, SE7-6PYE and SE7-6PYEαFPHO. Both strains contain plasmids, however, the PYEαFPHO plasmid has the ability to secrete acid phosphatase. Since the PYEαFPHO strain contains this special plasmid, which is more complex than the plasmid in the PYE, it takes longer to grow. During the last year, I have measured growth rates and secretion rates for both strains and I have also tried to optimize these processes as well.

## SURVEY OF LITERATURE

Due to the fact that my project consisted of many experiments that were microbiological in nature, I had to learn many new lab techniques. Most of the theory and microbiology techniques that were needed to complete my experiments were supplied by Dr. K. D. Wittrup. Late in the 1989 fall semester I began to learn these techniques. I learned how to pour plates, streak cell onto the plates, inoculate colonies into solution, use a Klett meter, and perform growth curves.

One important technique that I learned and used again and again was how to perform growth curves. As I mentioned above, I worked with two strains of yeast, and in order to grow the yeast it needs a growth medium with sufficient nutrients such as nitrogen, dextrose, and casamino acids. I would have to first streak both strains of yeast cells onto plates that contain these nutrients in a gel form, then place the plates in a 30 degree Celsius incubator for a few days until single colonies formed. These colonies would then be inoculated into a regular solution containing nutrients (SD-CAA). This solution is allowed to grow for 24 hours in a constant temperature aerator and is called an overnight. Then 0.1 ml of the PYE overnight and 0.25ml of the PYEαFPHO overnight are inoculated into a side arm flask that contains 50ml of SD-CAA. These solutions are placed in a 30 degree shaker and measurements of the optical density can be taken on a Klett meter. By plotting ln(Klett) verses time you obtain a growth curve.

Growth curves were used in one way or another in almost every experiment I ran, however, the only useful part of the growth curve is the exponential growth phase. When ln(Klett) is plotted verses time there is usually an eight to twelve hour lag phase while the cells adjust to the

medium and begin to multiply. The exponential growth phase usually starts at a Klett reading of 25 and can continue to a Klett reading of 200 until the yeast cells begin to level off. The slope of the line in the exponential phase corresponds to the growth rate of the cells. Many things can affect the growth rate such as: concentration of casamino acids and pH of the solution.

Once an optimal growth rate is achieved then the secretion rate of acid phosphatase can be measured. As yeast cells secrete acid phosphatase, some of the acid phosphatase stays inside the cells, some is found outside the cell walls (in the growth medium) and some is found attached to the cell wall. An assay was developed to measure the amount of acid phosphatase secreted inside the cell and in the growth medium. Unfortunately a method for determining the amount of acid phosphatase attached to the cell wall was attempted but never successfully achieved.

The assay I used to determine the secretion rate of acid phosphatase takes advantage of the fact that once acid phosphatase is produced it can be used to catalyze the reaction:

p-nitrophenyl phosphate -----> p-nitrophenol + phosphate
(yellow) (colorless)

The rate of reaction can then be determined by measuring the absorbance of the product at 435nm. This procedure will be explained in greater detail in the procedure section.

I used the above mentioned techniques and theory to study the effects of adding varying amounts of tunicamycin to the growth medium. Tunicamycin inhibits the addition of carbohydrates to the protein. Adding tunicamycin is important because carbohydrates can attach to many proteins, assist in protein folding, and have an effect on secretion.

## APPARATUS AND PROCEDURE

During the last year, I did not work on one main project, but rather four smaller projects that related to one another.

The first project I worked on was determining the optimal amount of casamino acids (CAA) to add to the growth medium (SD-CAA). In order to determine the optimal amount of CAA to add to the growth medium I had to measure growth curves of both strains of yeast at various concentrations of CAA. I began by first inoculating a single colony of each strain into 4ml of growth medium that contained 0% CAA. I grew these cultures for 24 hours (they were my overnights), and then inoculated ten side arm flasks (two of each) containing 0, 0.1, 0.5, 1.0 and 2.0% CAA with each yeast strain. I then measured the optical density of each sample with a Klett meter and plotted ln(Klett) versus time to obtain the growth curve for each sample. This same exact procedure was performed a second time to check the consistency of my results (see figures 1 - 4).

I also worked on measuring the plasmid-containing fraction of colonies by two separate methods. The first involved transferring colonies from YPD (non-selective) to SD-CAA (selective) plates. I performed this test by inoculating a single colony into 4ml of SD-CAA, letting it grow for 24 hours and then inoculating about 0.25ml of the overnight into a side arm flask (just like I was performing a growth curve). After a few hours I measured the Klett of the sample, used the correlation that one Klett approximately equals 60,000 cells/ml, and diluted the solution so I could transfer 500 cells to a YPD plate. All colonies grow on YPD plates because these plates are non-selective, however, only colonies which contain the plasmid will grow on the SD-CAA plates. Once the colonies grew on the YPD plates I had to transfer each colony

by hand to the SD-CAA plates, smear them out and wait to see which ones grew. Approximately 500 colonies had to be transferred to give a good statistical representation of the plasmid containing fraction. I performed this assay for PYE twice and for PYEαFPHO three times, for a total of 2500 colonies transferred all together.

It is important to calculate the plasmid-containing fraction of colonies because this number is needed in correcting average specific enzyme activities to a per-plasmid-containing-cell basis. However, the standard procedure for determining the plasmid-containing fraction that I described above is very tedious and time consuming. In order to save time and make life a little easier while performing enzyme assays I developed a new assay for measuring the plasmid-containing fraction.

The new assay involves growing approximately 500 cells on high phosphate YPD plates to suppress the native acid phosphatase in the cells. The recipe for approximately 40 plates is as follows:

| | |
|---|---|
| Bacto-yeast extract | 10.0g |
| Bacto-peptone | 20.0g |
| dextrose | 20.0g |
| Bacto-agar | 20.0g |
| $KH_2PO_4$ | 5.0g |
| Dilute to 1000ml | |

The cells are transferred to the high phosphate plates in the same manner as they were to the regular YPD plates in the previously described experiment. However, once the colonies grew on these plates I poured an agar solution containing α-napthyl phosphate and fast garnet over the colonies instead of transferring each cell. The theory behind this method is that the two reactants added to the agar solution react with any acid phosphatase present to

form a red precipitate. So in theory any colonies that contain the acid phosphatase producing plasmid will turn red once the agar solution is poured over them.

In order to obtain the most readable results I performed this assay with varying amount of each reactant. A step by step assay protocol follows:

For each plate to be covered it takes approximately 8-10ml of agar.
Make a 1.5% agar solution with 50mM acetate buffer with a pH of 4.
Melt the solution by using a hot plate or an autoclave.
Cool the solution to 50°C in a water bath.
Add α-naphthyl phosphate and fast garnet to desired concentrations.
Be certain all reactants are dissolved.
Pour agar solution over the colonies on the high-phosphate YPD plates.
Do not move the plates until the agar has set.
Place plates in the incubator for at least one hour to be sure all reactants have reacted.
Use the colony counter to count the number of colonies that turned red and the ones that did not, mark each colony as you count it with a tooth pick.

The third project I worked on involved determining the acid phosphatase activity in the yeast cells and in the growth medium. This was done by inoculating an overnight sample of the yeast strain (either PYE or PYEαPHO) into a side arm flask and a taking a sample at a desired Klett reading. The cells are then centrifuged out and the supernatant is saved. The cells are resuspended in a 50mM acetate buffer to a Klett of 50 (where $Klett_1(volume)_1 = Klett_2(volume)_2$). Then at time = 0, 50μL of each sample

(resuspended cells and supernatant) is added to 0.6mL of acetate buffer containing 2.0mg/ml p-nitrophenyl phosphate at 30°C. This is the assay that was described earlier in which the secreted acid phosphatase from the yeast cells catalyzes the reaction:

p-nitrophenyl phosphate (clear) -----> p-nitrophenol + phosphate (yellow).

The reaction is run for a total of twenty minutes with samples taken at ten and twenty minutes (two samples at each time are taken for both the supernatant and the whole cells). The reaction is terminated by placing the microfuge tubes on ice and adding 0.15 ml of 25% TCA and 0.7ml of a saturated $Na_2CO_3$ solution. The samples are then placed in cuvettes and the absorbance of the sample is taken at a wavelength of 435nm in a spectrophotometer. The activity of the enzyme can be determined by taking a least squares fit of the absorbance data.

I completed two full sets of activity measurements (taken at Klett readings from 25 to 100) for both PYE and PYEαFPHO during the spring 1990 semester (taken from slants) and one set of PYE and two sets of PYEαFPHO during the fall 1990 semester (taken from cold storage). I took four Klett readings for each sample and for each Klett reading I used the least squares method to determine the slope of the points (which is equal to the activity). I then calculated the specific activity for the cells ($AP_s$) by dividing the activity by the (Klett x sample volume) and the specific activity for the supernatant ($AP_o$) by dividing the activity by the volume of the supernatant (see Table I for results from slants and figures 6-9 and Table V for results from cold storage).

After the specific activities were calculated, the rate of production of total external acid phosphatase ($V_p$) and the rate of secretion of acid phosphatase out of the cell to the medium ($V_s$) were calculated. This was done by

numerically evaluating the integral $\int(AP_s/N)dN$ (N = Klett) which equals $\int(V_p-V_s)dt$. The integral was evaluated using the trapezoidal rule and taking a Klett of 25 equal to a time of zero. A similar integral was then evaluated ($\int(dAP_o/N) = \int V_s dt$ ) using the same assumptions and techniques. These newly obtained integrals were then plotted against time and the slope was calculated to obtain ($V_p - V_s$ ) and $V_s$ respectively. From these values it was easy to obtain $V_p$ (see Tables II and III for results from slants and Table V and VI as well as figures 10 - 13 for results from cold storage).

The last project I worked on was determining the toxicity curve for tunicamycin and how different concentrations of tunicamycin effect the secretion of acid phosphatase in yeast. I inoculated 0.05 ml of an overnight of both strains of yeast into test tubes containing 5 ml of SD-CAA solution with 0, 1.0µg/ml, 10µg/ml, 50µg/ml, and 100µg/ml of tunicamycin. I allowed these test tubes to grow for 24 hours in the 30°C shaker and then measured the Klett at t=24 hours. I also took samples of each culture (only at t = 24 hours) to perform the enzyme assay to determine how tunicamycin effects the secretion rate of acid phosphatase (see Table IV and figure 5).

After determining the toxicity level for tunicamycin, I then performed the regular enzyme assay (in the shake flask from klett = 25 to 100) on various samples containing tunicamycin. This stage of my research was worked on only in the fall 1990 semester and used strains of yeast taken from cold storage only. Since it was determined the previous semester that very small amounts of tunicamycin (10µg/ml) kill the yeast cells, tests were performed by adding 1µg/ml of tunicamycin (see figure 9, Table V, figure 13, and Table VI for results).

## RESULTS AND DISCUSSION

As I mentioned above, the results from the growth curves with varying amounts of casamino acids are presented in figures 1 through 4. The graphs of ln(Klett) versus time are plotted for times only in the exponential growth range, so these graphs give straight lines with equations of the form Y=b + mX, where m=slope and it also equals the growth rate. We need to use a concentration of CAA that gives the greatest growth rate and by looking at all the graphs it seems that a concentration of 0.5% CAA gives the greatest growth rate in three out of four trials. This value also happens to be the standard concentration used by other researchers.

From the results determined above and the results of John Flahive's pH tests, the standard recipe for SD-CAA is as follows:

| | |
|---|---|
| Dextrose | 20.0g/l |
| Sodium Citrate | 14.7g/l |
| Yeast Nitrogen Base | 6.6g/l |
| Casamino Acids | 5.0g/l |
| Citric Acid Monohydrate | 4.2g/l |

The data collected from the first method of determining the plasmid-containing fraction of colonies (the transferring of colonies from YPD to SD-CAA) is presented below.

| Type of Strain | Trial # | % Containing Plasmid |
|---|---|---|
| PYEαFPHO | 1 | 4.9 |
| PYEαFPHO | 2 | 5.8 |
| PYEαFPHO | 3 | 8.0 |
| PYE | 1 | 97.7 |
| PYE | 2 | 97.7 |

This data is very consistent for the PYE strain and clearly indicates that almost all the colonies contain the plasmid. However, this is not the plasmid which produces acid phosphatase. The plasmid in the PYEαFPHO strain is the plasmid that has the ability to secrete acid phosphatase. Unfortunately, my data for this strain is not very consistent and indicates that 4.9 to 8.0% of the colonies may contain the plasmid. I tend to trust the third trial more than the first two because in the first two trials I transferred around 200 colonies onto one SD-CAA plate, hence the colonies were very crowded and did not have much room to grow. In trial three I only transferred about 70 colonies onto a plate and the colonies in this trial had more room to grow and as a result it was much easier to tell which colonies grew and which did not.

My results for the second method of determining the plasmid containing fraction of colonies are presented below for PYEαFPHO. They were obtained by simultaneously spreading out 6 plates of colonies on high phosphate YPD and pouring solutions of agar that contain 1, 2 and 4mg/l of α-naphthyl phosphate and 1 and 2 mg/ml of fast garnet over them.

| α (mg/ml) | garnet (mg/ml) | % Containing Plasmid |
|---|---|---|
| 1 | 1 | 9.01 |
| 2 | 1 | 12.45 |
| 4 | 1 | 9.80 |
| 1 | 2 | 8.94 |
| 2 | 2 | 8.57 |
| 4 | 2 | 8.62 |

These results are relatively consistent with each other and with the data for trial #3 of PYEαFPHO in the first assay. The six different plates were tested to find out which combination of reactants would give the most readable results. The plates with 2mg/ml of fast garnet seemed to be the easiest to read so these were tested again along with three plates containing the PYE strain to see if the assay was giving any false results (there should be no acid phosphatase produced in PYE therefore no red precipitate should appear). The results of these tests indicated that the combination of 2mg/ml of α-naphthyl phosphate and 2mg/ml of fast garnet gave the most readable results and the tests with PYE indicated that the assay was not giving any false results.

The results from my third project, the secretion rates of acid phosphatase, are located in Tables I through VI and in figures 6 through 13. Table III contains my final results from the experiments performed with slants (spring 1990) and Table VI contains my final results from the fall 1990 (cold storage cells). By looking at Table III you can see that two sets of my data have negative secretion rates. This implies that the cells do not produce acid phosphatase but instead consume it. My results are very inconsistent with each other and when they were compared with Dan Pack's (also from spring 1990), only one rate was consistent between the two sets.

| | PYE | PYE | PYEαFPHO | PYEαFPHO |
|---|---|---|---|---|
| | $V_p$ ($\times 10^5$) | $V_s$ ($\times 10^5$) | $V_p$ ($\times 10^5$) | $V_s$ ($\times 10^5$) |
| K.O. | .9 | .8 | 2.3 | .3 |
| D.P. | -.9 | -.003 | 2.2 | 1.1 |

note: The secretion rates ($V_p$ and $V_s$) are averaged values.

Comparing my results from Table III (slants) and Table VI (cold storage) there seems to be little difference between the values. One noticeable improvement in the samples from cold storage is that all the secretion rates are positive. However, values for $V_p$ and $V_s$ are on the average, some what lower for the samples taken from cold storage.

The results from my initial experiments with tunicamycin are presented in Table IV and figure 5. Table IV gives the Klett, concentration of tunicamycin added, and the specific activities of the supernatant and whole cells. If you look at figure 5, which is a plot of the Klett versus the ln(concentration of tunicamycin) at time = 24 hours, you can see that as the concentration of tunicamycin increases the Klett decreases until it reaches a some what constant value. The slight increase in Klett at the two highest concentrations of tunicamycin could be due to the fact that tunicamycin is not very soluble in SD-CAA at high concentrations so the solutions became cloudy from the tunicamycin not from yeast cells.

The graphs in figure 5 should start off at a high Klett number at low concentrations of tunicamycin and then begin decreasing in Klett as tunicamycin is added. At the concentrations of tunicamycin where the Klett begins to decrease is where the specific activity should begin to increase. The specific activity should continue to increase for awhile and then decrease until it reaches a minimum corresponding to the point where the Klett levels off again. Unfortunately, my data for $AP_s$ and $AP_0$ for PYEαFPHO do not seem to agree with what I expected.

At the beginning of the fall 1990 semester I decided to narrow my range of added tunicamycin. I decided to begin with a concentration of 1μg/ml of tunicamycin. Since time was limited due to problems with reviving the cells

from cold storage in the beginning of semester, I did not perform another toxicity curve but rather began performing full scale assays. Unfortunately, due to many unforeseen problems only one complete experiment was completed (results can be seen in figure 9, Table V, figure 13, and Table VI). The values for $V_p$ and $V_s$ were found to be an order of magnitude smaller when this small amount of tunicamycin was added. Even though another complete set of data was not taken the same results were observed when the experiment was duplicated. Decreases in secretion were also observed for a concentration of 0.5 µg/ml of tunicamycin. Therefore, while adding tunicamycin may assist in protein folding it definitely decreases the secretion rate of acid phosphatase from yeast.

## CONCLUSIONS AND RECOMMENDATIONS

My results for determining the optimal amount of CAA to add to the growth medium are quite consistent and suggest that a concentration of 0.5% CAA will allow the yeast cells to grow at the fastest rate. My results for the new assay to determine the plasmid-containing fraction are also consistent and suggest that a concentration of 2mg/ml of fast garnet and 2mg/ml of α-naphthyl phosphate will give the most readable results. However, the results from my other two experiments are not as well defined.

One reason for the inconsistency in the secretion rates in the experiments performed from slants (spring 1990) could be due to the cuvettes that were used in the spectrophotometer. The cuvettes wer disposable microliter cuvettes. When I recorded the absorbance data on the spectrophotometer, I would turn the cuvettes around 180°, take the spectrum again. and noticed

that the results would change by as much as 50%. This problem was corrected for my experiments ran in the fall of 1990 (cells from cold storage) by using milliliter cuvettes. My results for these experiments as a result, were more consistent with one another and no negative secretion rates were observed as in the experiments performed a semester earlier.

As for my results concerning the addition of tunicamycin, it was discovered that adding any amount of tunicamycin decreases the secretion rate and by adding 10 μg/ml of tunicamycin or more you kill the yeast cells all together. I would therefore recommend that studies of the protein folding be done for added concentrations of tunicamycin ranging from 0 μg/ml to 10 μg/ml.

The results of these experiments are important to the medical world. If the growth medium is manipulated to allow the yeast cell to produce the optimal amount of protein, this process can be set up at a larger scale to commercially produce the protein.

## ACKNOWLEDGEMENTS

I would like to thank Dr. K. D. Wittrup for all his help and guidance in preparing this thesis.

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# APPENDIX

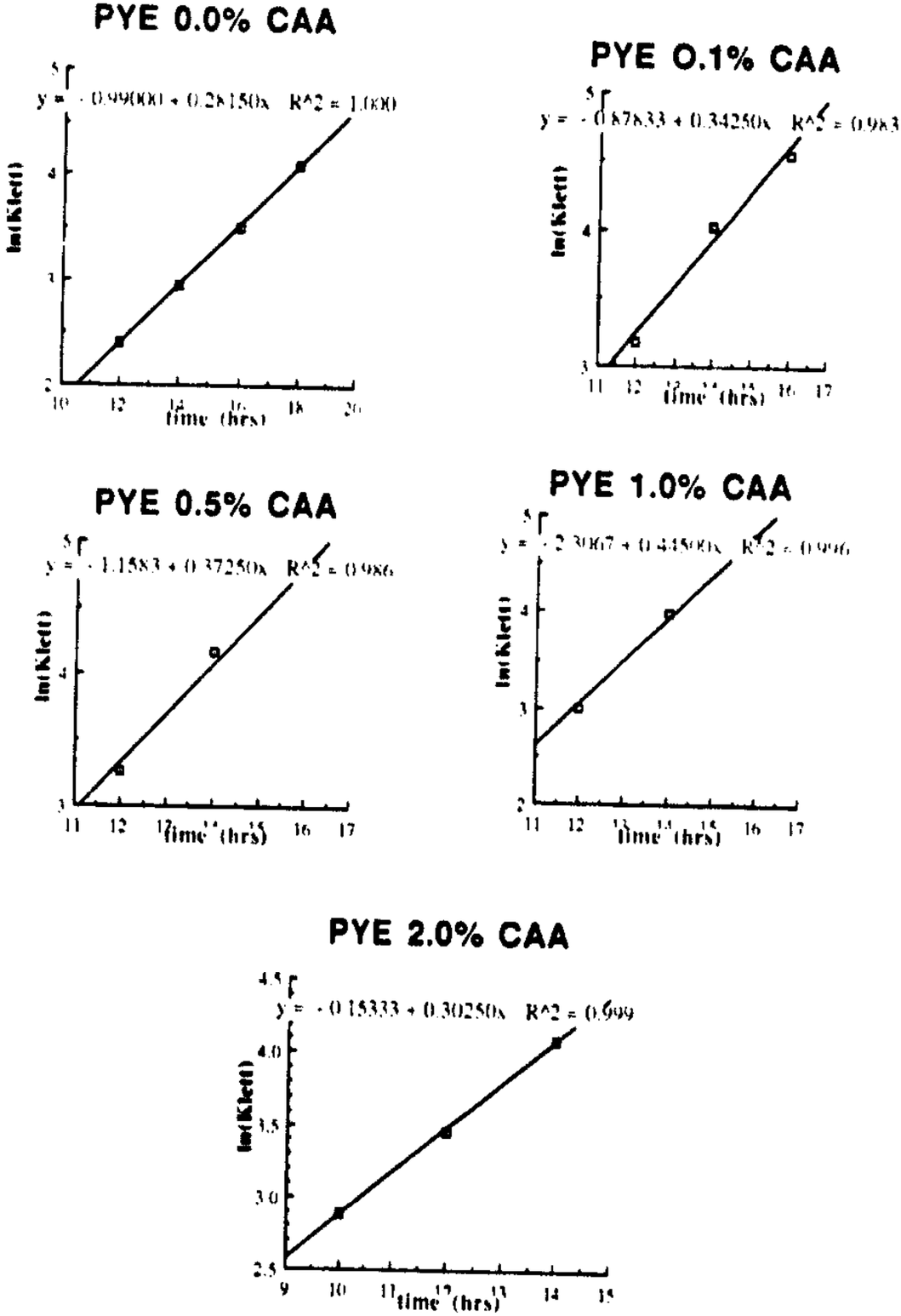



Figure 1 Pye Trial #1

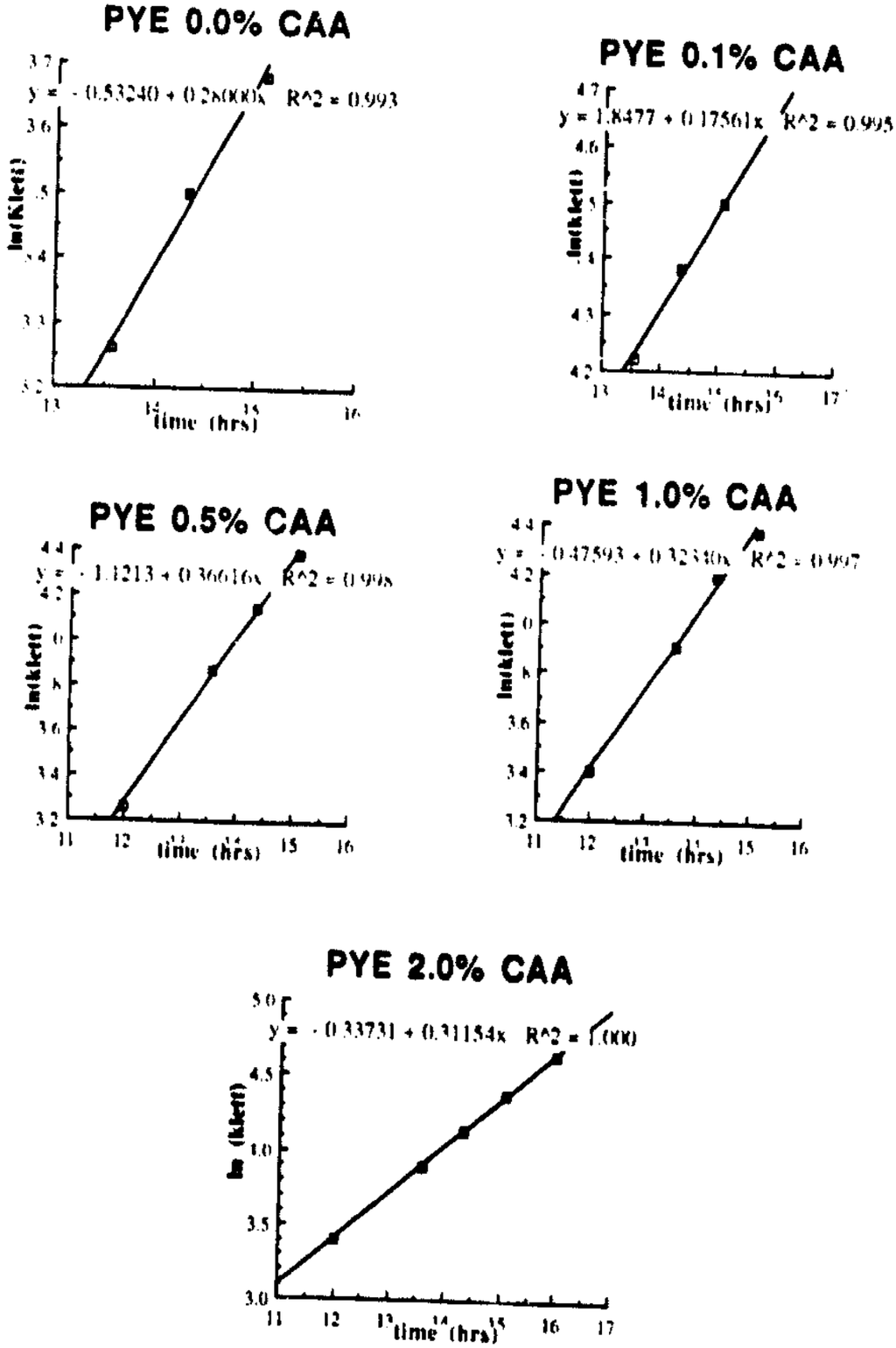
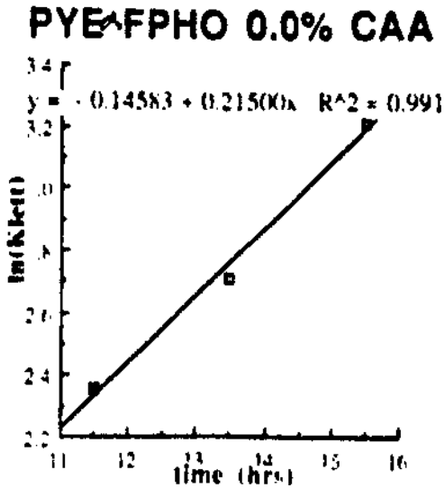
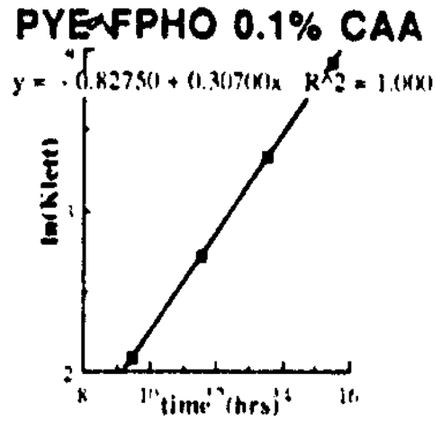
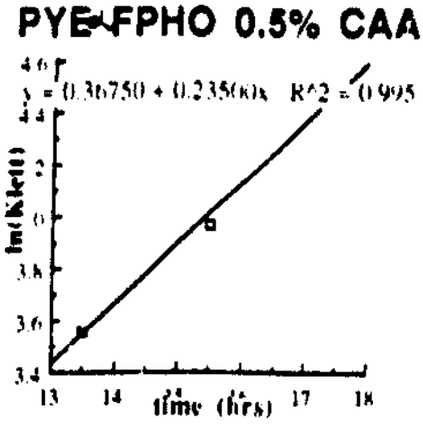
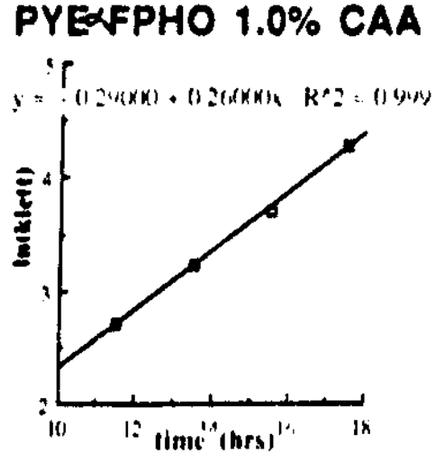



Figure 2 Pye Trial #2



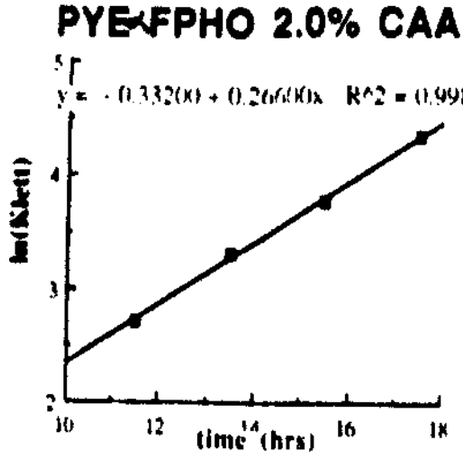
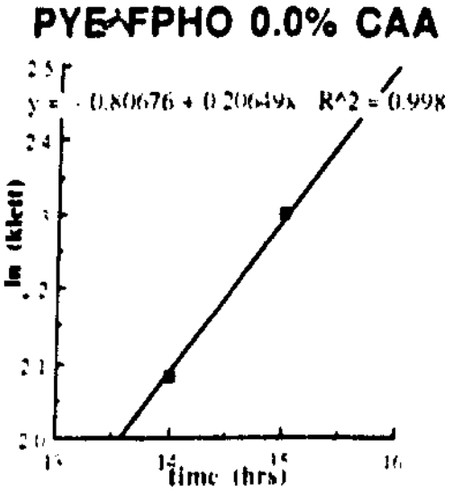
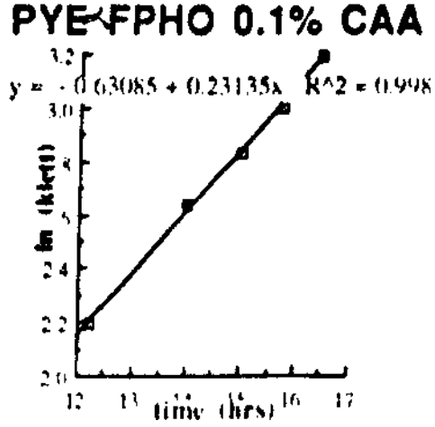
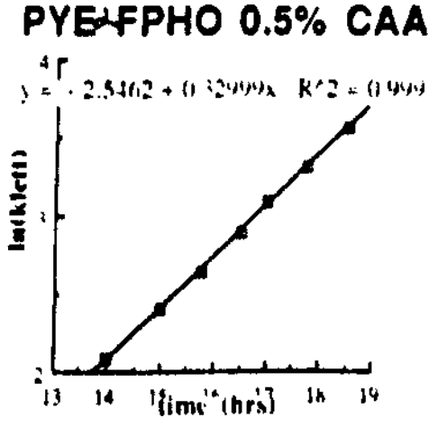
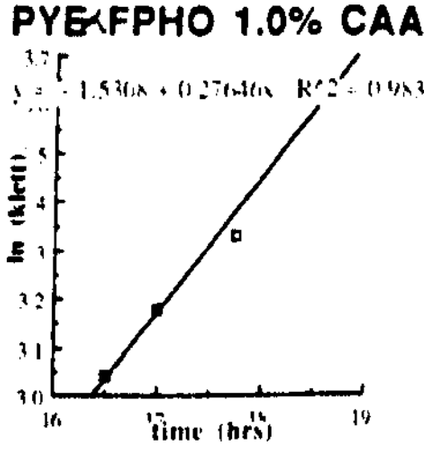




Figure 3 PyeαFPHO Trial #1



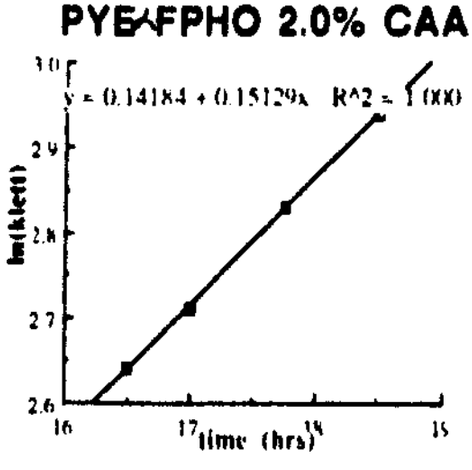




Figure 4 PyeAFPHO trial #2

Table I

| Trial #1 | Klett | time (min) | $A_{Ps}$ ($min^{-1}mL^{-1}Klett^{-1}$) | $A_{Po}$ ($min^{-1}mL^{-1}$) |
|---|---|---|---|---|
| PYE | 25 | 0 | .00182 | .03700 |
| PYE | 50 | 105 | -.00028 | -.01200 |
| PYE | 75 | 180 | .00046 | .06180 |
| PYE | 100 | 240 | .00010 | .16700 |
| Trial #2 | | | | |
| PYE | 25 | 0 | .00038 | .01300 |
| PYE | 50 | 105 | .00012 | .02400 |
| PYE | 75 | 195 | -.00022 | -.07800 |
| PYE | 100 | 255 | .00080 | -.04800 |
| Trial #1 | | | | |
| PYEαFPHO | 25 | 0 | .00418 | .19200 |
| PYEαFPHO | 60 | 210 | .01044 | .31000 |
| PYEαFPHO | 75 | 300 | .00824 | .31200 |
| PYEαFPHO | 100 | 390 | .00822 | .29580 |
| Trial #2 | | | | |
| PYEαFPHO | 25 | 0 | .00148 | .05200 |
| PYEαFPHO | 50 | 210 | .00376 | .15900 |
| PYEαFPHO | 75 | 345 | .00720 | .28300 |
| PYEαFPHO | 100 | 495 | .00416 | .28600 |

Table II

| Trial #1 | Klett | time (min) | $\int(Vp-Vs)dt$ ($min^{-1}mL^{-1}Klett^{-1}$) | $\int Vsdt$ ($min^{-1}mL^{-1}Klett^{-1}$) |
|---|---|---|---|---|
| PYE | 25 | 0 | | |
| PYE | 50 | 105 | $8.39x10^{-4}$ | $-1.47x10^{-3}$ |
| PYE | 75 | 180 | $8.46x10^{-4}$ | $-2.40x10^{-4}$ |
| PYE | 100 | 240 | $9.35x10^{-4}$ | $9.87x10^{-4}$ |
| Trial #2 | | | | |
| PYE | 25 | 0 | | |
| PYE | 50 | 105 | $2.20x10^{-4}$ | $3.30x10^{-4}$ |
| PYE | 75 | 195 | $2.13x10^{-4}$ | $-1.37x10^{-3}$ |
| PYE | 100 | 255 | $2.76x10^{-4}$ | $-1.02x10^{-3}$ |
| Trial #1 | | | | |
| PYEαFPHO | 25 | 0 | | |
| PYEαFPHO | 60 | 210 | $5.97x10^{-3}$ | $3.34x10^{-3}$ |
| PYEαFPHO | 75 | 300 | $8.10x10^{-3}$ | $3.37x10^{-3}$ |
| PYEαFPHO | 100 | 390 | .0105 | $3.18x10^{-3}$ |
| Trial #2 | | | | |
| PYEαFPHO | 25 | 0 | | |
| PYEαFPHO | 50 | 210 | $1.68x10^{-3}$ | $3.21x10^{-3}$ |
| PYEαFPHO | 75 | 345 | $3.82x10^{-3}$ | $5.28x10^{-3}$ |
| PYEαFPHO | 100 | 495 | $5.54x10^{-3}$ | $5.32x10^{-3}$ |

Table III
(final results)

PYE Trial #1

$V_p - V_s = 6.97 \times 10^{-7}$ (activity/min)/cell

$V_p$ = rate of production of total external AP = $2.74 \times 10^{-5}$ (activity/min)/cell

$V_s$ = rate of secretion of AP out of cell to medium = $2.67 \times 10^{-5}$ (activity/min)/cell

PYE Trial #2

$V_p - V_s = 3.41 \times 10^{-7}$ (activity/min)/cell

$V_p = -9.42 \times 10^{-6}$ (activity/min)/cell

$V_s = -9.76 \times 10^{-6}$ (activity/min)/cell

PYE$\alpha$FPHO Trial #1

$V_p - V_s = 2.52 \times 10^{-5}$ (activity/min)/cell

$V_p = 2.43 \times 10^{-5}$ (activity/min)/cell

$V_s = -8.65 \times 10^{-7}$ (activity/min)/cell

PYE$\alpha$FPHO Trial #2

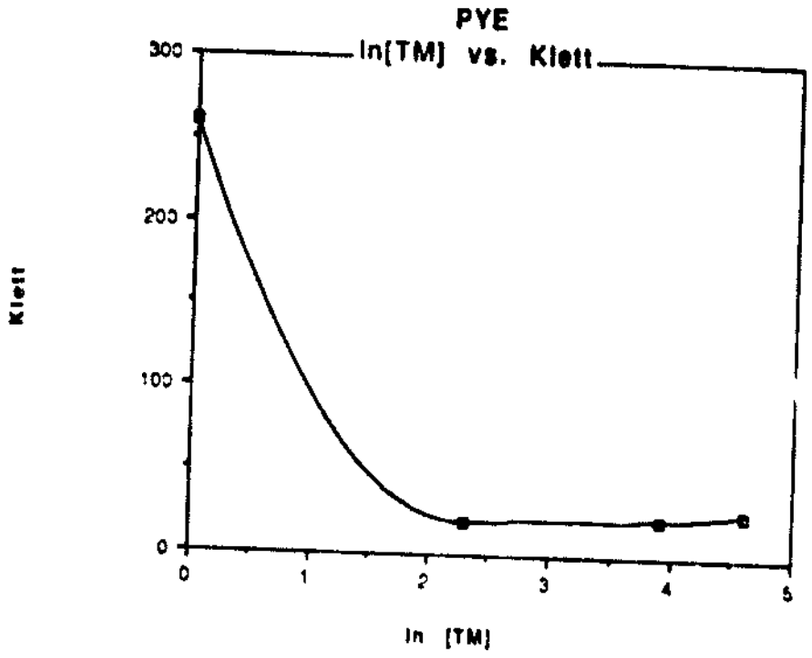
$V_p - V_s = 1.35 \times 10^{-5}$ (activity/min)/cell

$V_p = 2.08 \times 10^{-5}$ (activity/min)/cell

$V_s = 7.28 \times 10^{-6}$ (activity/min)/cell

Table IV

| | [TM] (μg/mL) | Klett | APs ($min^{-1}mL^{-1}Klett^{-1}$) | APo ($min^{-1}mL^{-1}$) |
|---|---|---|---|---|
| PYE | 0.0 | 423 | $-9.2x10^{-4}$ | $1.0x10^{-3}$ |
| PYE | 1.0 | 260 | $6.8x10^{-4}$ | 0.019 |
| PYE | 10.0 | 20 | $1.0x10^{-3}$ | 0.021 |
| PYE | 50.0 | 22 | | |
| PYE | 100.0 | 27 | | |
| PYEαFPHO | 0.0 | 200 | $6.0x10^{-3}$ | 0.575 |
| PYEαFPHO | 1.0 | 50 | $1.5x10^{-3}$ | 0.089 |
| PYEαFPHO | 10.0 | 3 | $1.8x10^{-3}$ | 0.026 |
| PYEαFPHO | 50.0 | 4 | | |
| PYEαFPHO | 100.0 | 8 | | |


PYE
ln[TM] vs. Klett
300
200
100
0
Klett
0
1
2
3
4
5
ln [TM]

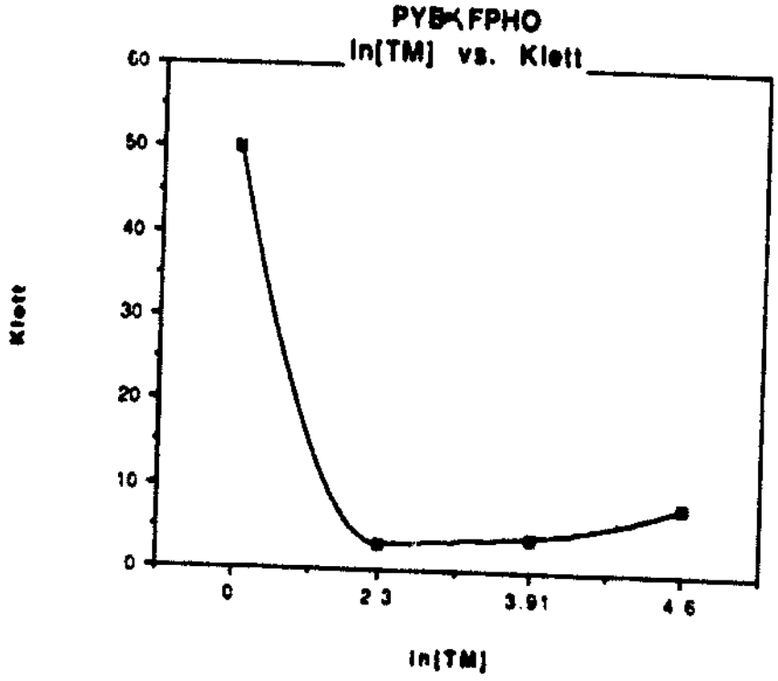
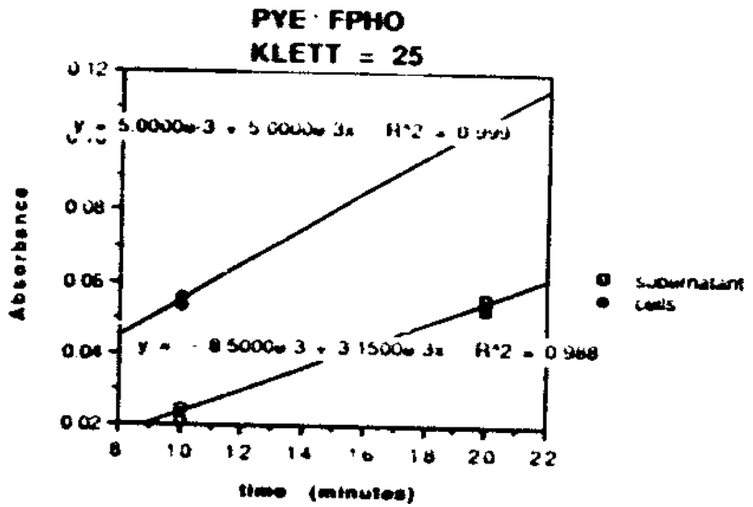
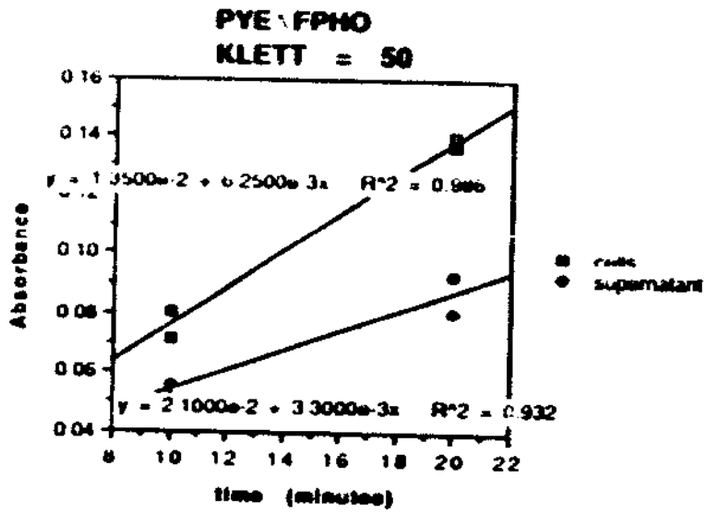
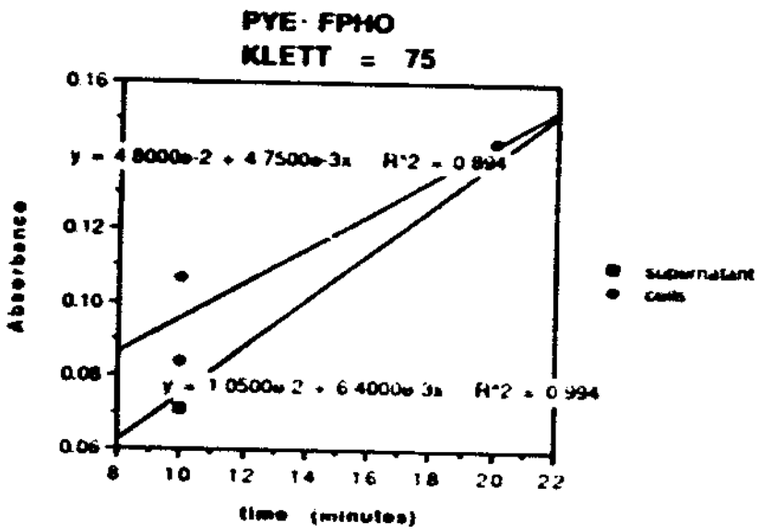

PYE-FPHO
ln[TM] vs. Klett
60
50
40
30
20
10
0
Klett
0
2.3
3.91
4.6
ln[TM]

Figure 5


PYE · FPHO
KLETT = 25
Absorbance
time (minutes)
supernatant
cells


PYE · FPHO
KLETT = 50
Absorbance
time (minutes)
supernatant


PYE · FPHO
KLETT = 75
y = 4.8000e-2 + 4.7500e-3x R^2 = 0.894
y = 1.0500e-2 + 6.4000e-3x R^2 = 0.994
Absorbance
time (minutes)

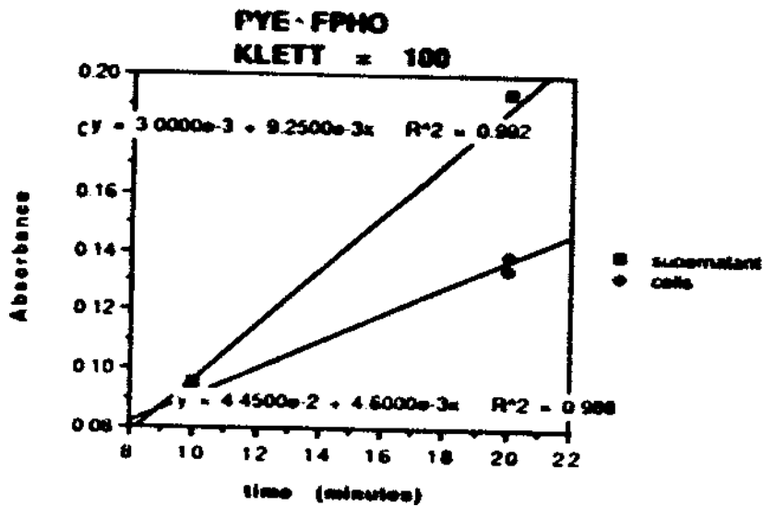

PYE · FPHO
KLETT = 100
y = 3.0000e-3 + 9.2500e-3x R^2 = 0.992
y = 4.4500e-2 + 4.6000e-3x R^2 = 0.988
Absorbance
time (minutes)

Figure 6

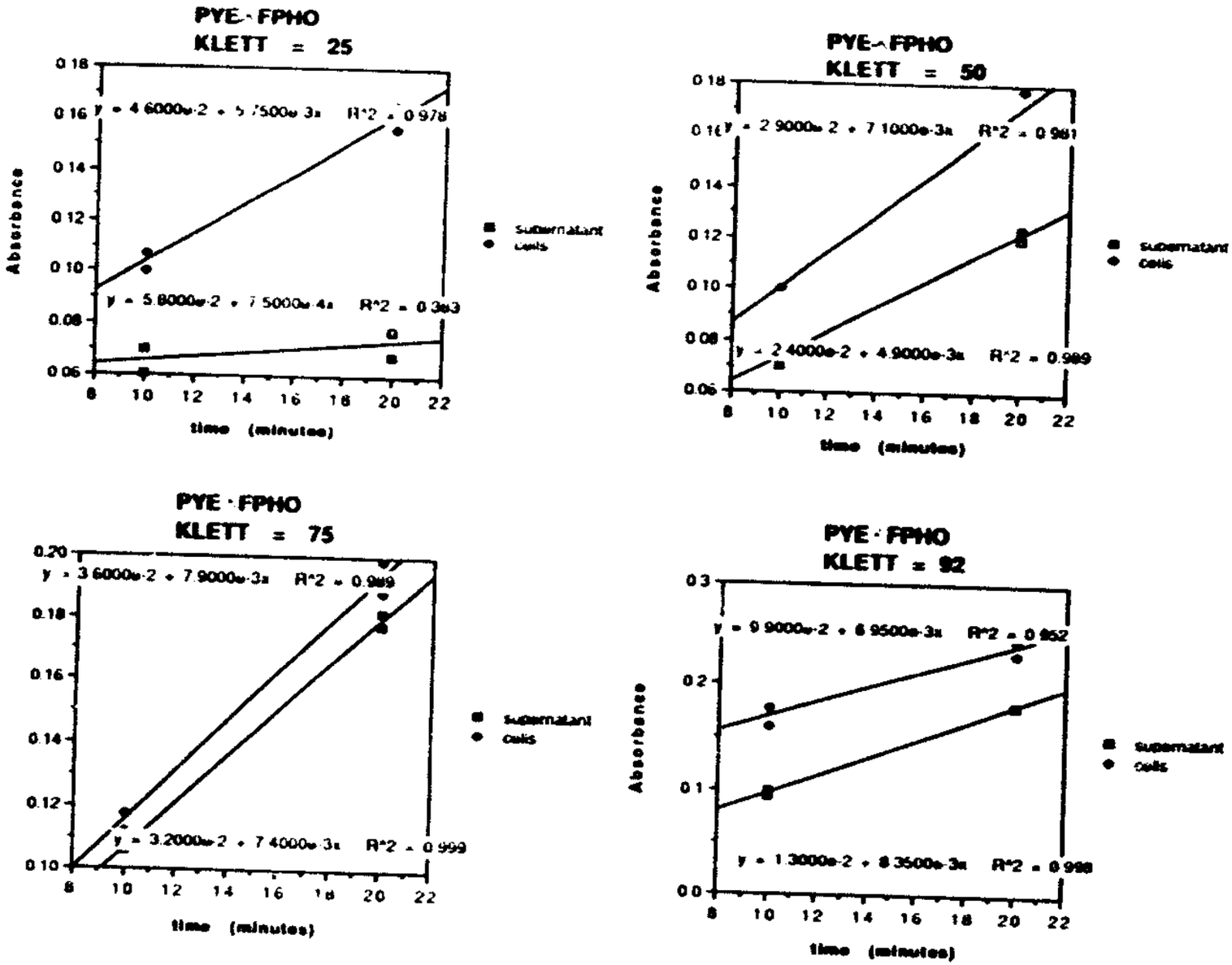

PYE - FPHO
KLETT = 25
y = 4.6000e-2 + 5.7500e-3x R^2 = 0.978
y = 5.8000e-2 + 7.5000e-4x R^2 = 0.383
Absorbance
time (minutes)
supernatant
cells
PYE - FPHO
KLETT = 50
y = 2.9000e-2 + 7.1000e-3x R^2 = 0.981
y = 2.4000e-2 + 4.9000e-3x R^2 = 0.989
Absorbance
time (minutes)
supernatant
cells
PYE - FPHO
KLETT = 75
y = 3.6000e-2 + 7.9000e-3x R^2 = 0.989
y = 3.2000e-2 + 7.4000e-3x R^2 = 0.999
Absorbance
time (minutes)
supernatant
cells
PYE - FPHO
KLETT = 92
y = 9.9000e-2 + 6.9500e-3x R^2 = 0.952
y = 1.3000e-2 + 8.3500e-3x R^2 = 0.988
Absorbance
time (minutes)
supernatant
cells

Figure 7

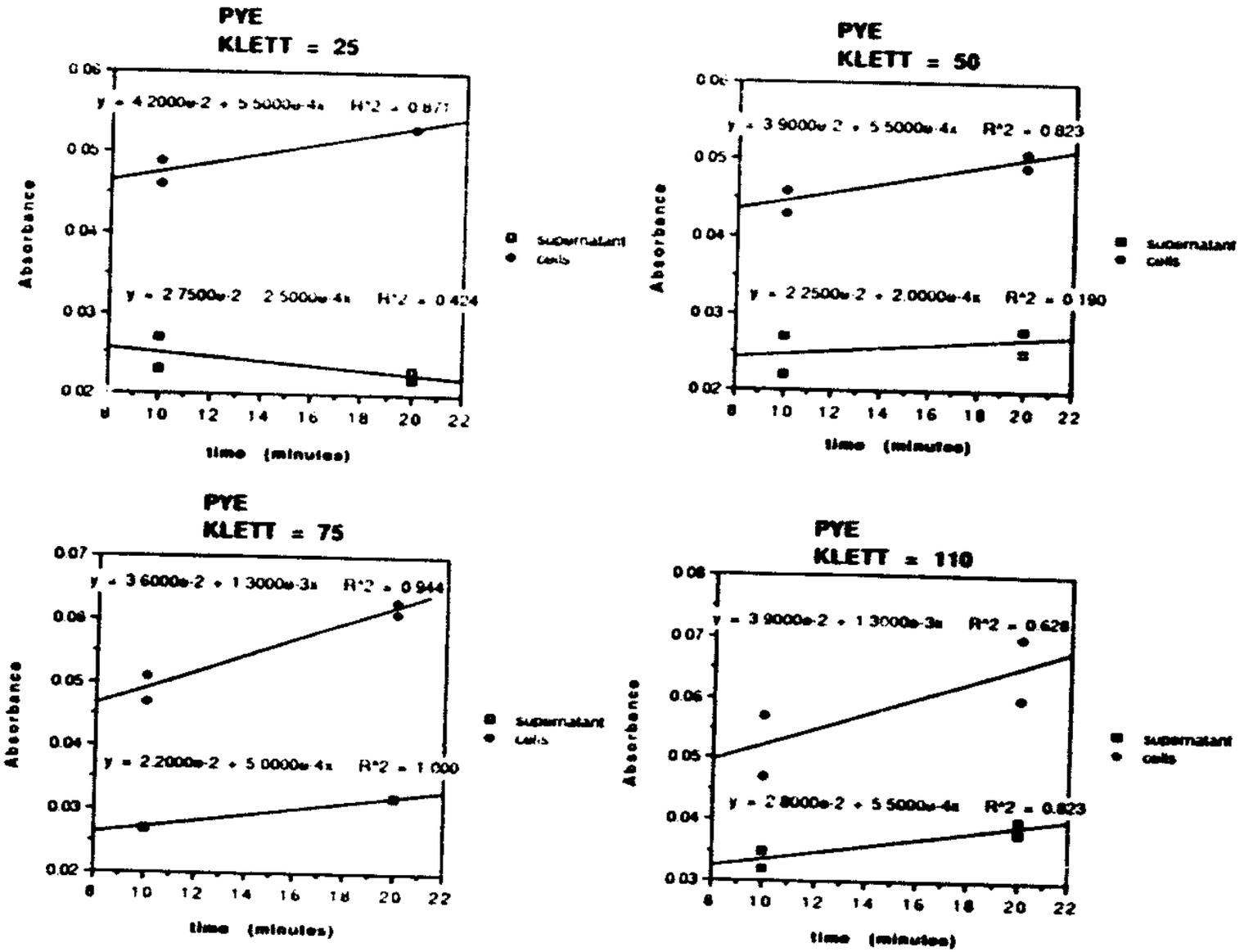
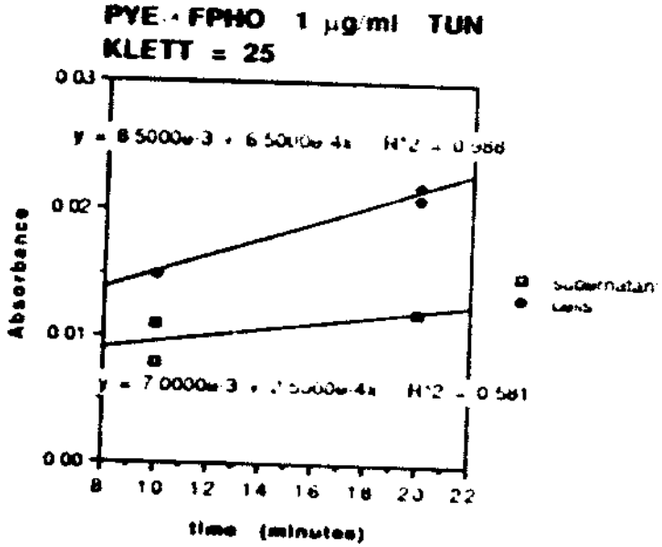
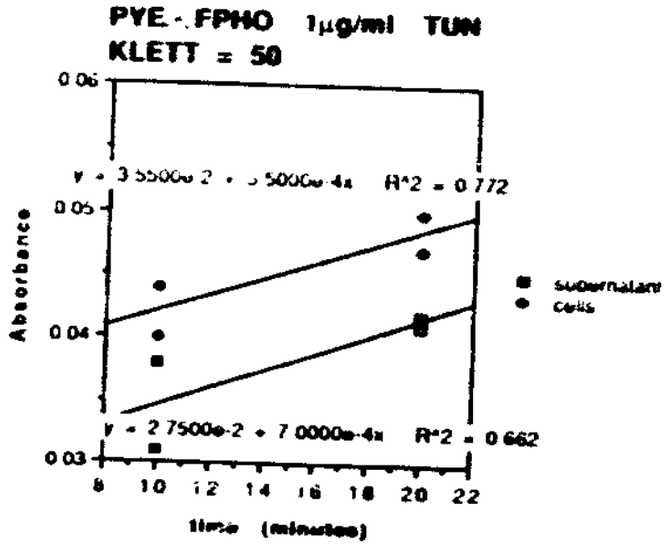

PYE
KLETT = 25
y = 4.2000e-2 + 5.5000e-4x R^2 = 0.877
y = 2.7500e-2 2.5000e-4x R^2 = 0.424
supernatant
cells
Absorbance
time (minutes)
PYE
KLETT = 50
y = 3.9000e-2 + 5.5000e-4x R^2 = 0.823
y = 2.2500e-2 + 2.0000e-4x R^2 = 0.190
supernatant
cells
Absorbance
time (minutes)
PYE
KLETT = 75
y = 3.6000e-2 + 1.3000e-3x R^2 = 0.944
y = 2.2000e-2 + 5.0000e-4x R^2 = 1.000
supernatant
cells
Absorbance
time (minutes)
PYE
KLETT = 110
y = 3.9000e-2 + 1.3000e-3x R^2 = 0.628
y = 2.8000e-2 + 5.5000e-4x R^2 = 0.823
supernatant
cells
Absorbance
time (minutes)

Figure 8


PYE - FPHO 1 µg/ml TUN
KLETT = 25
Absorbance
time (minutes)
supernatant
cells


PYE - FPHO 1µg/ml TUN
KLETT = 50
Absorbance
time (minutes)
supernatant
cells

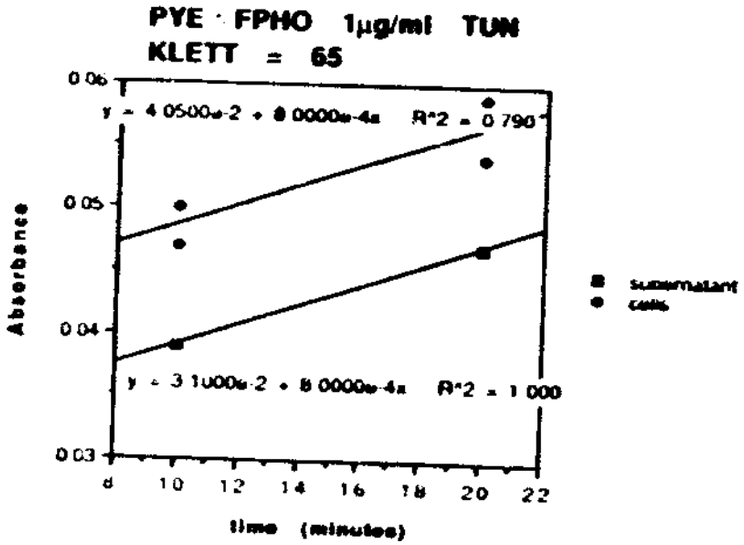
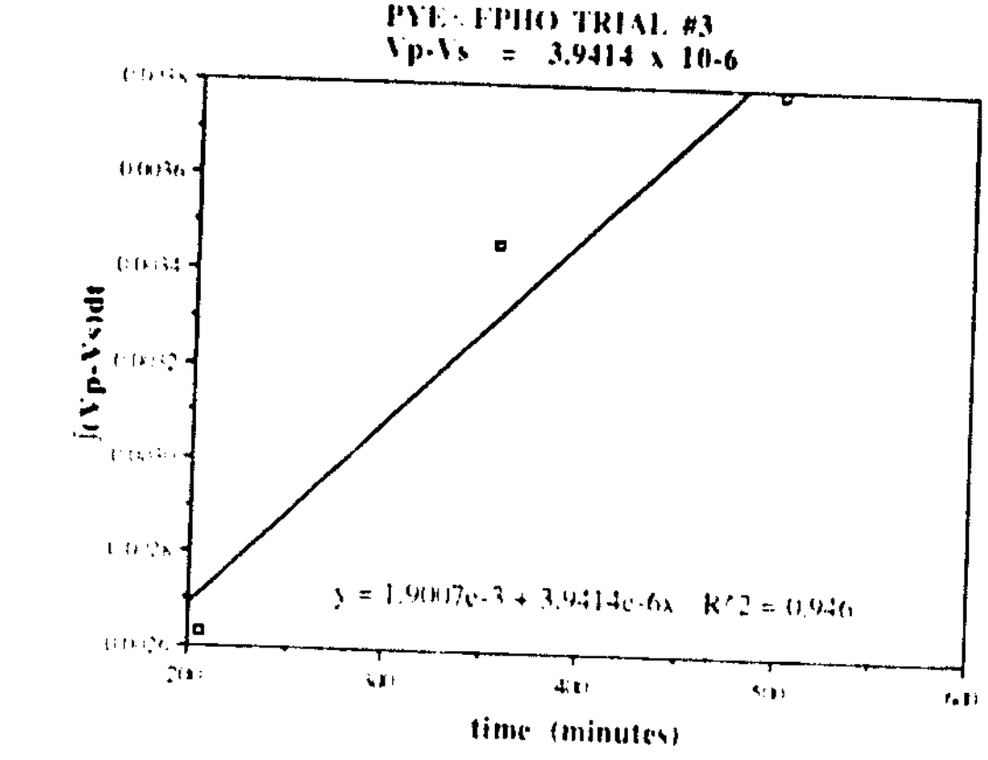

PYE - FPHO 1µg/ml TUN
KLETT = 65
Absorbance
time (minutes)
supernatant
cells

Figure 9

| | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| 1 | | | | Table V | | | | |
| 2 | | | | | | | | |
| 3 | KLETT | TIME (MIN) | SAMPLE | CELLS | APo | APs | ∫(Vp Vs)dt | ∫Vsdt |
| 4 | | | | | | | | |
| 5 | PYE+FPHO #3 | | | | | | | |
| 6 | 25 | 0 | 0.00315 | 0.00500 | 0.06300 | 0.00400 | 0.00000 | 0.00000 |
| 7 | 50 | 235 | 0.00330 | 0.00625 | 0.06600 | 0.00250 | 0.00263 | 0.00009 |
| 8 | 75 | 355 | 0.00640 | 0.00475 | 0.12800 | 0.00127 | 0.00346 | 0.00112 |
| 9 | 100 | 500 | 0.00924 | 0.00460 | 0.18500 | 0.00092 | 0.00379 | 0.00179 |
| 10 | | | | | | | | |
| 11 | PYE+FPHO #4 | | | | | | | |
| 12 | 25 | 0 | 0.00075 | 0.00575 | 0.01500 | 0.00460 | 0.00000 | 0.00000 |
| 13 | 50 | 235 | 0.00490 | 0.00710 | 0.09800 | 0.00284 | 0.00301 | 0.00249 |
| 14 | 75 | 390 | 0.00740 | 0.00790 | 0.14800 | 0.00211 | 0.00407 | 0.00332 |
| 15 | 92 | 510 | 0.00835 | 0.00695 | 0.16700 | 0.00151 | 0.00445 | 0.00355 |
| 16 | | | | | | | | |
| 17 | PYE #3 | | | | | | | |
| 18 | 25 | 0 | 0.00025 | 0.00055 | 0.00500 | 0.00044 | 0.00000 | 0.00000 |
| 19 | 50 | 95 | 0.00030 | 0.00055 | 0.00400 | 0.00022 | 0.00028 | 0.00027 |
| 20 | 75 | 150 | 0.00050 | 0.00130 | 0.01000 | 0.00035 | 0.00039 | 0.00037 |
| 21 | 110 | 240 | 0.00065 | 0.00130 | 0.01100 | 0.00024 | 0.00051 | 0.00038 |
| 22 | | | | | | | | |
| 23 | PYE+FPHO #5 1µg ml TUN | | | | | | | |
| 24 | 25 | 0 | 0.00025 | 0.00065 | 0.00500 | 0.00052 | 0.00000 | 0.00000 |
| 25 | 50 | 420 | 0.00040 | 0.00065 | 0.01400 | 0.00026 | 0.00033 | 0.00027 |
| 26 | 65 | 705 | 0.00060 | 0.00040 | 0.01600 | 0.00025 | 0.00039 | 0.00031 |


PYE-FPHO TRIAL #3
Vp-Vs = 3.9414 x 10-6
∫(Vp-Vs)dt
y = 1.9007e-3 + 3.9414e-6x R^2 = 0.946
time (minutes)

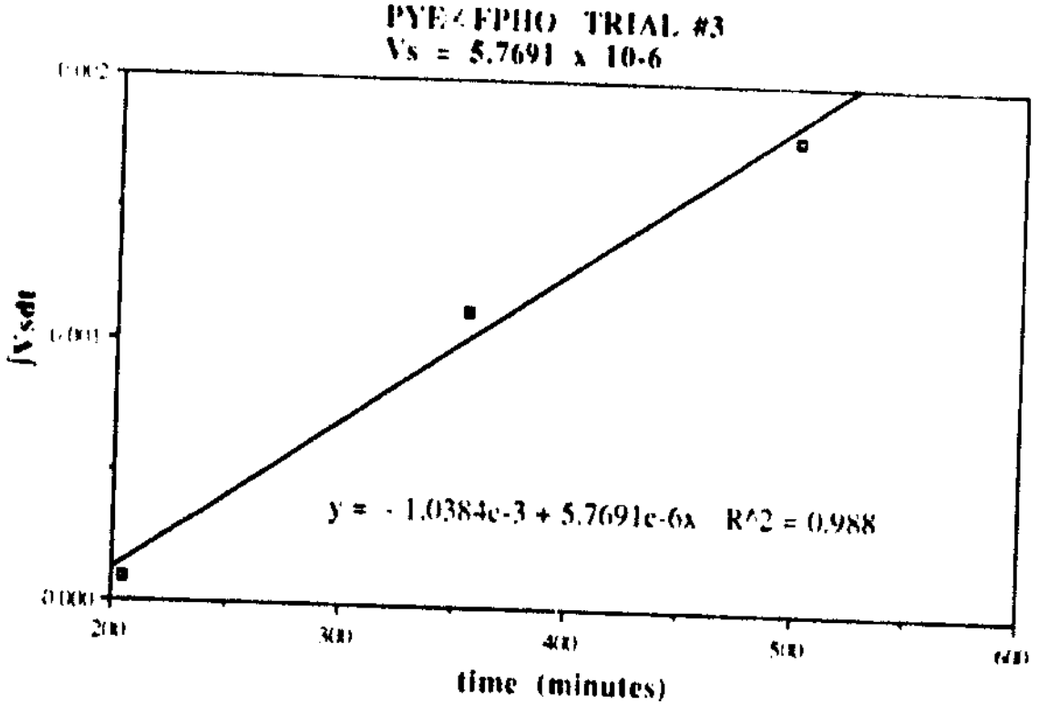
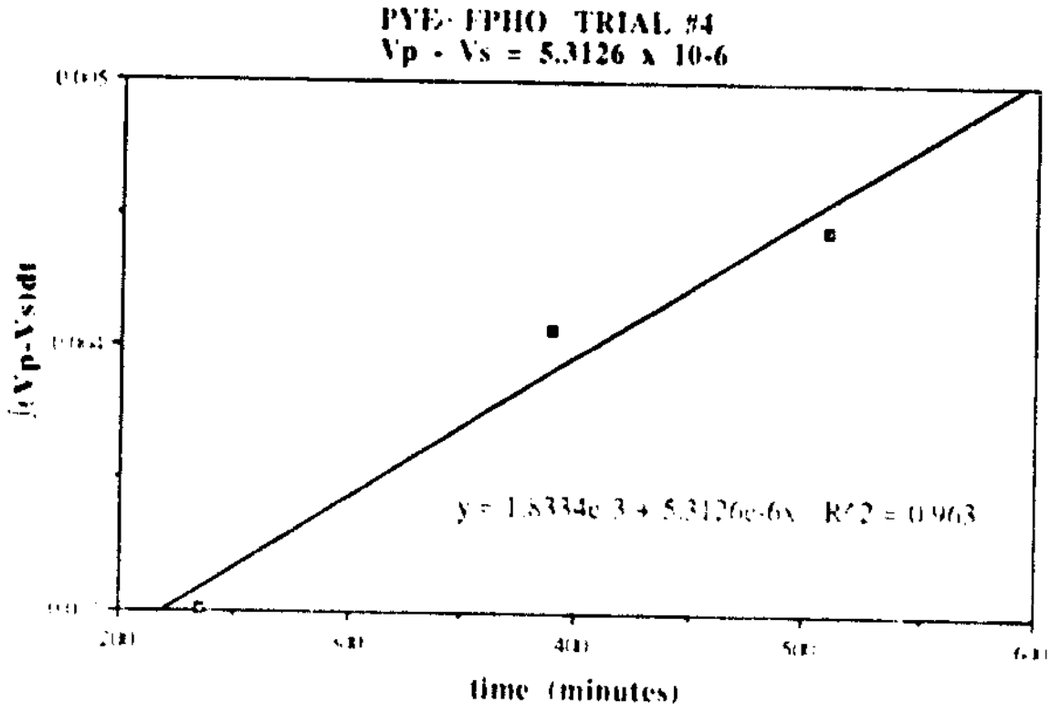

PYE-FPHO TRIAL #3
Vs = 5.7691 x 10-6
∫Vsdt
y = -1.0384e-3 + 5.7691e-6x R^2 = 0.988
time (minutes)

Figure 10


PYE- FPHO TRIAL #4
Vp - Vs = 5.3126 x 10-6
∫(Vp-Vs)dt
y = 1.8334e-3 + 5.3126e-6x R^2 = 0.963
time (minutes)

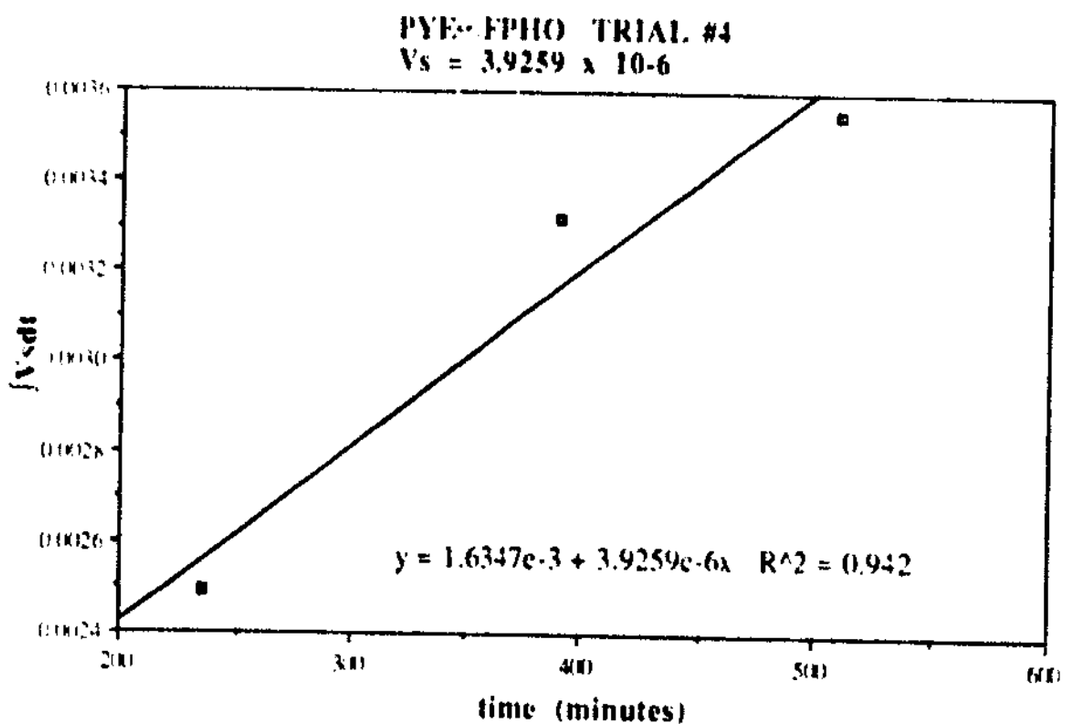
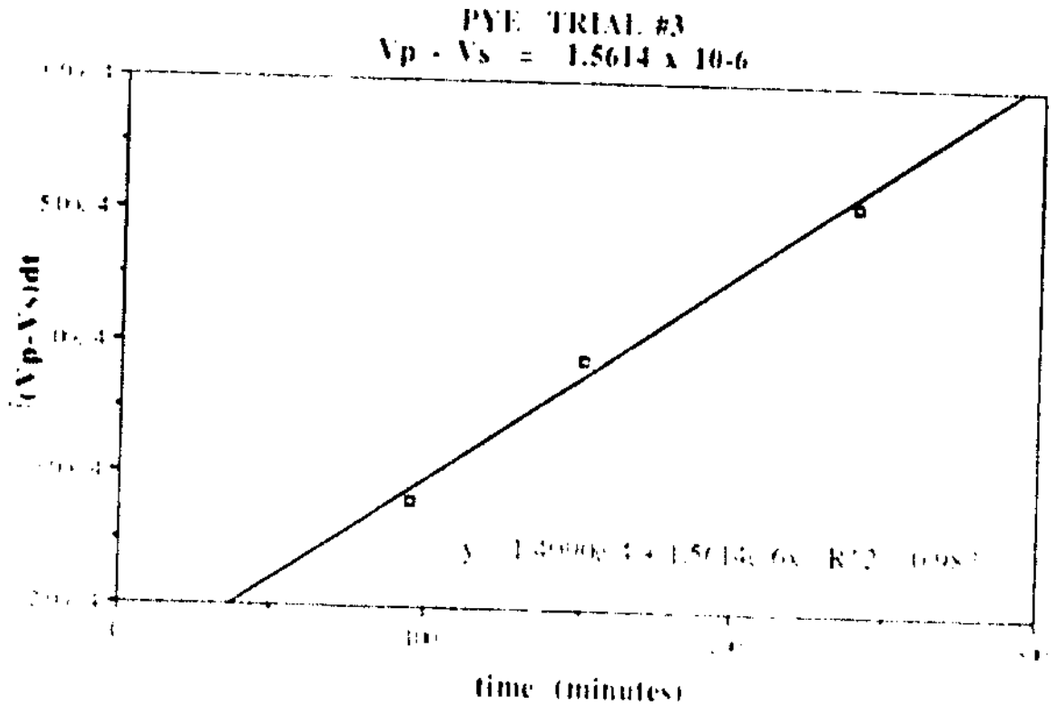

PYE- FPHO TRIAL #4
Vs = 3.9259 x 10-6
∫Vsdt
y = 1.6347e-3 + 3.9259e-6x R^2 = 0.942
time (minutes)

Figure 11


PYE TRIAL #3
Vp - Vs = 1.5614 x 10-6
∫(Vp-Vs)dt
time (minutes)

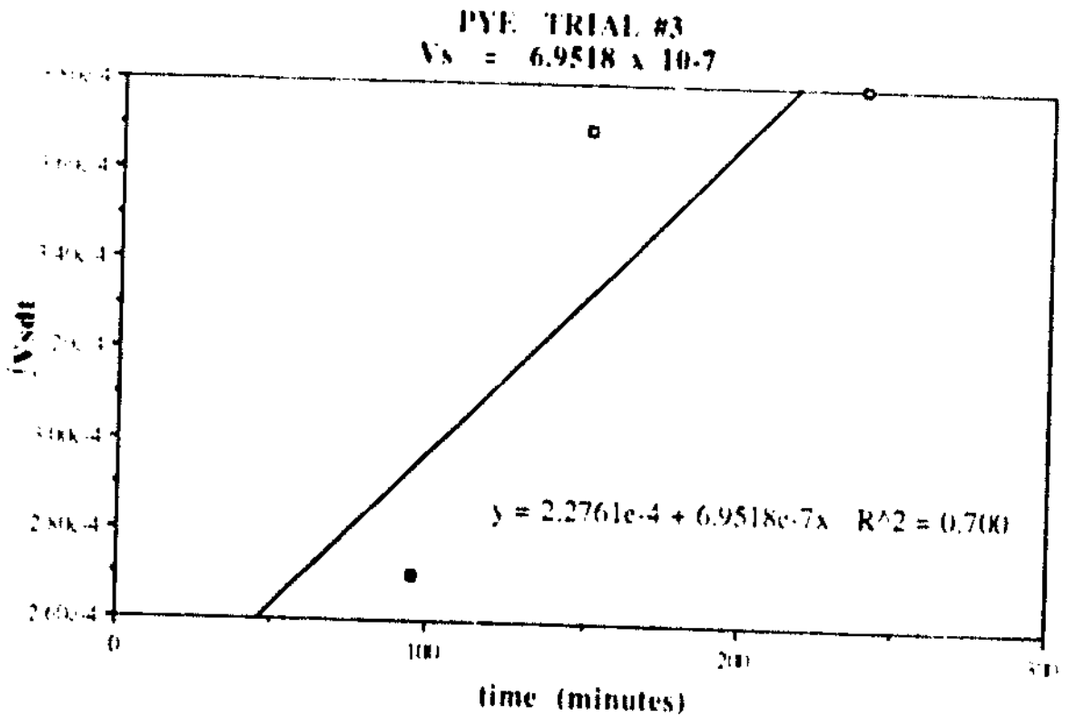
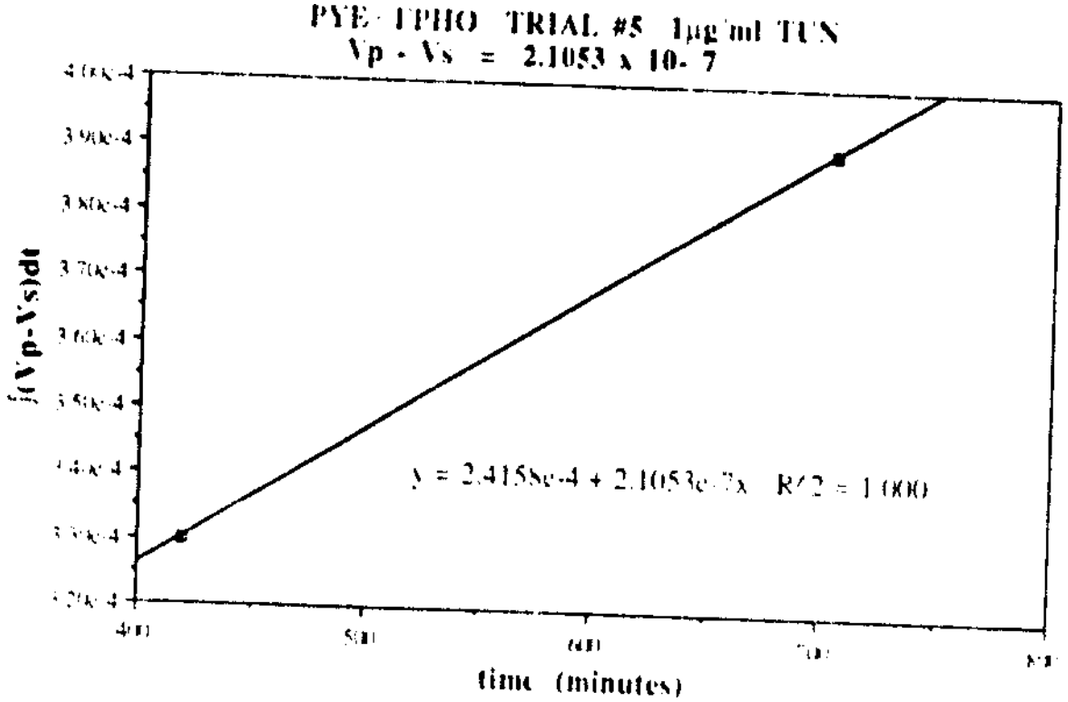

PYE TRIAL #3
Vs = 6.9518 x 10-7
∫Vsdt
y = 2.2761e-4 + 6.9518e-7x R^2 = 0.700
0
100
200
time (minutes)

Figure 12


PYE · FPHO TRIAL #5 1µg/ml TUN
Vp - Vs = 2.1053 x 10- 7
∫(Vp-Vs)dt
y = 2.4158e-4 + 2.1053e-7x R^2 = 1.000
time (minutes)

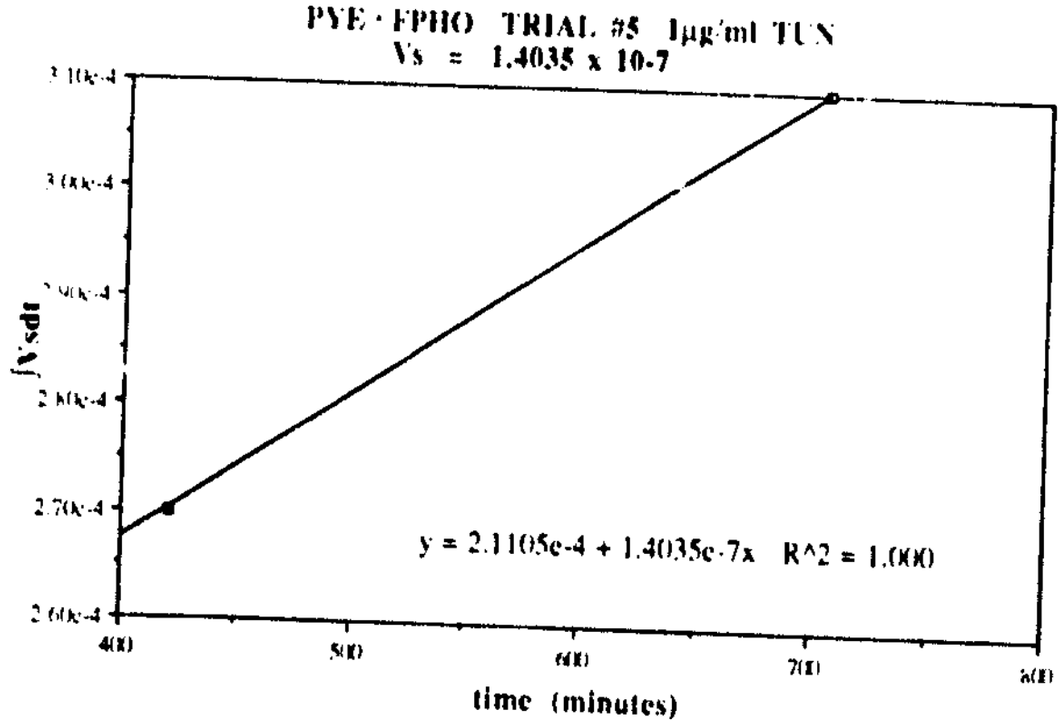

PYE · FPHO TRIAL #5 1µg/ml TUN
Vs = 1.4035 x 10-7
∫Vsdt
y = 2.1105e-4 + 1.4035e-7x R^2 = 1.000
4(0)
5(0)
6(0)
7(0)
8(0)
time (minutes)

Figure 13

Table VI
(final results from cold storage)

## PYE Trial #3

$V_p - V_s = 1.5614 \times 10^{-6}$ (activity/min)/cell

$V_p$ = rate of production of total external AP = $2.2566 \times 10^{-6}$ (activity/min)/cell

$V_s$ = rate of secretion of AP out of cell to medium = $6.9518 \times 10^{-7}$ (activity/min)/cell

## PYEαFPHQ Trial #3

$V_p - V_s = 3.9414 \times 10^{-6}$ (activity/min)/cell

$V_p = 9.7105 \times 10^{-6}$ (activity/min)/cell

$V_s = 5.7691 \times 10^{-6}$ (activity/min)/cell

## PYEαFPHQ Trial #4

$V_p - V_s = 5.3126 \times 10^{-6}$ (activity/min)/cell

$V_p = 9.2385 \times 10^{-6}$ (activity/min)/cell

$V_s = 3.9259 \times 10^{-6}$ (activity/min)/cell

## PYEαFPHQ Trial #5
(1µg/ml TUN)

$V_p - V_s = 2.1053 \times 10^{-7}$ (activity/min)/cell

$V_p = 3.5088 \times 10^{-7}$ (activity/min)/cell

$V_s = 1.4035 \times 10^{-7}$ (activity/min)/cell