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Viscosity and gravity effects on the kinetics of E. coli alkaline phosphatase

Sujatha Muralidharan

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by

Sujatha Muralidharan

Undergraduate honors thesis under the direction of

Dr. Vince LiCata

Department of Biological Sciences

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Louisiana State University
& Agricultural and Mechanical College
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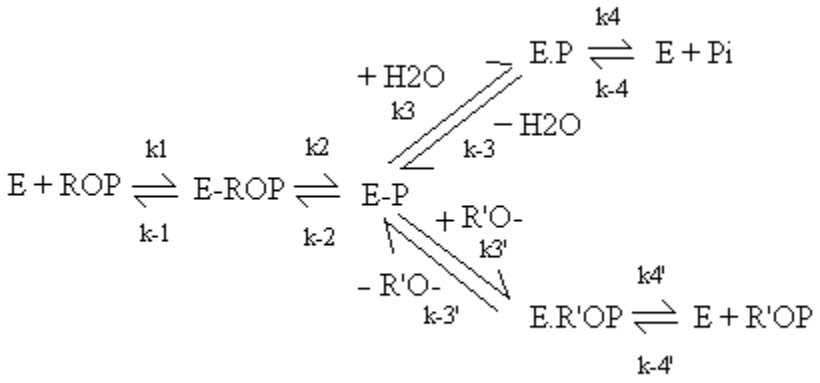
ABSTRACT:

In order to determine if alkaline phosphatase is diffusion controlled, the kinetics of the enzyme was characterized as a function of glycerol viscosity. The effect of microgravity on enzyme kinetics was also investigated on NASA's C9 airplane during parabolic flights that simulate microgravity. Since human alkaline phosphatase plays a role in bone mineralization, the results of these microgravity AP kinetics experiments may help in explaining the abnormal bone mineralization seen in astronauts after extensive spaceflight. The reaction used to study the kinetics of alkaline phosphatase was enzyme catalyzed removal of phosphate from para-nitrophenyl phosphate. The experiments were conducted in stopped flow apparatus and the data obtained was fit for initial velocity and analyzed with the Michaelis Menten equation. The results show a decrease in V_{max} and V_{max}/K_m with increasing viscosity, which indicates diffusion control. The microgravity experiment results show no recognizable pattern of change in the kinetic parameters as a function of gravity.

INTRODUCTION:

Alkaline phosphatase (AP) is an enzyme that catalyzes the removal of a phosphate group from phosphate monoesters such as AMP and α-glycerol phosphate through hydrolysis. The reaction mechanism is as follows:



Scheme 1 [Modified from 1]

where E is the enzyme alkaline phosphatase and ROP is the substrate, a phosphate monoester. The enzyme substrate complex E-ROP is converted into a phosphoenzyme intermediate E-P and RO^- is released. Free enzyme is regenerated through hydrolysis of the phosphoenzyme to release inorganic phosphate Pi or by transfer of the phosphate group to another phosphate acceptor, $R'O^-$ [2].

The reaction of para-nitrophenyl phosphate (pNPP) and the enzyme *E. coli* alkaline phosphatase was used for the experiments in this study (Figure A). The product of the reaction, para-nitrophenol is yellow in color and the absorbance can be detected at 410 nm (Figure B). The Michelis Menten equation was used to analyze the enzyme kinetics.

$$V = \frac{V_{max}\ [S]}{[S] + K_m}$$

where V_{max} is the maximum velocity of the reaction and K_m is the Michaelis constant. [S] denotes the substrate concentration, which in this case is the pNPP concentration. The K_m is the substrate concentration at which the reaction rate is half the maximum value, ($½V_{max)}$ and it is a measure of the strength of binding between enzyme and substrate. V_{max} is the maximum velocity attained when the enzyme catalytic sites are saturated with substrate.

$$V_{max} = k_{cat} [E]$$

It reveals the turnover number of the enzyme (k_{cat}), which is the number of molecules of substrate converted to product by one enzyme site per unit time, if the concentration of active enzyme is known. These parameters K_m and V_{max} describe the kinetics of the enzyme [3].

The catalytic efficiency of the reaction was investigated using viscosity experiments. Enzymes that have attained kinetic perfection are called diffusion controlled enzymes. Their rate of reaction is limited only by the rate at which diffusion causes interaction of the enzyme with substrate in the solution. Therefore, viscosity experiments are a good indicator of the level of diffusion control in a reaction. If the reaction slows down due to increased viscosity, it suggests that the enzyme is diffusion controlled. Such viscosity experiments would enable us to determine if alkaline phosphatase is a diffusion controlled enzyme [3]. Glycerol was used for these viscosity experiments because it stabilizes the native structure of the protein by interacting unfavorably with non-polar groups in the interior of the protein. This ensures that any change in the rate of the reaction catalyzed by the enzyme is due to the viscosity and not due to destabilization or denaturation of the protein by the viscogen [4].

The effect of microgravity on diffusion controlled enzymes and reactions provide an interesting question. It is possible that the absence of gravity might decrease the rate of reaction by causing the molecules in solution to drift apart and move slower without the influence of gravitational force. This would increase time needed for the enzyme to encounter the substrate in the correct orientation thus reducing the rate of reaction. On the other hand, if an increased rate of reaction is observed in microgravity, it may be due to decreased convective mixing. The absence of the gravitational force reduces perturbations caused by convective mixing which may lead to more productive encounters between enzyme and substrate. It is also possible that microgravity has no effect on the rate of reaction at all.

This question was investigated with *E. coli* alkaline phosphatase by conducting microgravity experiments in the NASA C9 plane. The trajectory of the C9 plane in which environments of zero gravity and 1.8 times gravity are attained is shown in Figure C. The results of the experiment may have physiological relevance. It has been found that astronauts tend to have weaker bones after extensive spaceflight. These effects cannot be completely eliminated by exercise. Studies with rhesus monkeys have also shown that spaceflight causes problems with bone mineralization making them weaker. Human tissue non-specific alkaline phosphatase (TNAP) is found in the liver, kidney and bones. TNAP has been found to play a role in bone mineralization. Mutations in this enzyme have been associated with disorders characterized by defective bone mineralization. Thus any effect of microgravity on the reaction catalyzed by TNAP may also have some effect on bone mineralization. The effect of microgravity on enzymatic reactions such as that

catalyzed by alkaline phosphatase may have important implications for the responses of astronauts to long periods of residence in microgravity environments [5, 6, 7, 8].

In summary, the main goals of the study were to use viscosity to measure the degree of diffusion control of alkaline phosphatase and to determine the effect of microgravity on the enzymatic rate of the enzyme.

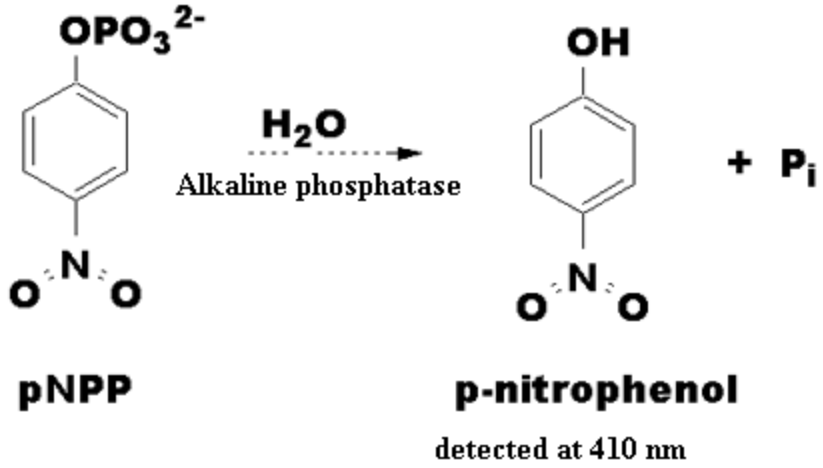


Figure A: Reaction catalyzed by *E. coli* alkaline phosphatase used for the experiments in this study

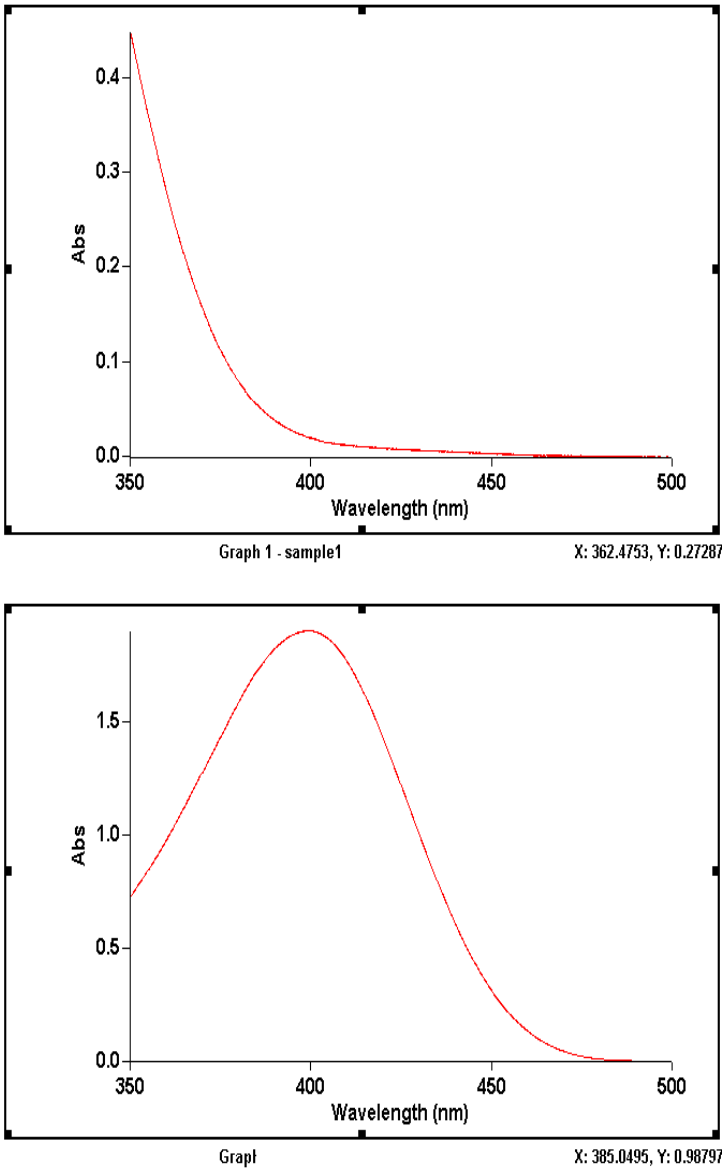


Figure B: Wavelength scans showing the difference in absorbance for a solution of pNPP (a) before and (b) after addition of enzyme

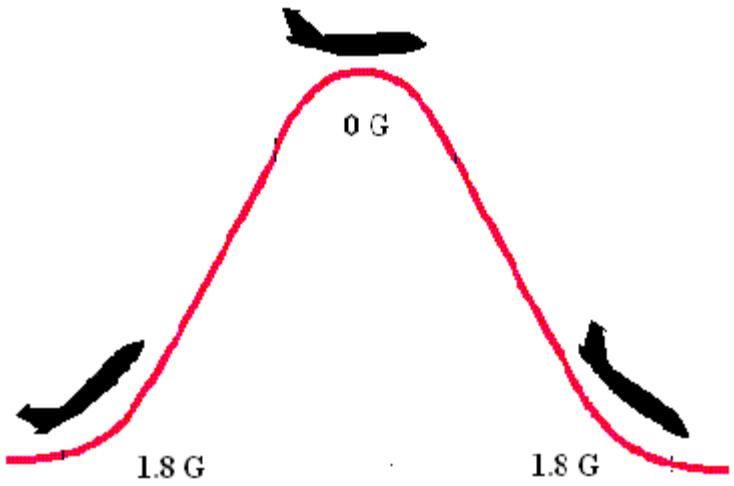


Figure C: Trajectory of NASA C9 plane in which conditions of 0 G and 1.8 G are attained (http://zerog.jsc.nasa.gov/home.html)

METHODS:

The AP-pNPP reaction was studied using stopped flow techniques. Stopped flow is a method for studying the kinetics of a reaction in which the two reactant solutions are mixed rapidly and then the flow of this mixed solution along a uniform tube is arrested. The solution can then be monitored as a function of time using some method with a rapid response such as spectrophotometry.

Figures D and E show photographs of the actual equipment set-up for the experiments. Two carts were used to hold all the instruments including an ISS Koala spectrofluorometer and a Bio-Logic SFM 3 stopped-flow. The spectrofluorometer was attached to the top of the bigger steel cart using the foot holes, which are normally used to attach the instrument to an optical bench. The stopped-flow was integrated with the spectrofluorometer in the center of the cart. A simple schematic of the components of the stopped-flow module is shown in Figure F. For the experiments, syringe 2 was filled with the substrate pNPP and *E. coli* alkaline phosphatase was loaded in syringe 3. During the experiments, small volumes of AP and pNPP were pushed out of syringes 2 and 3 through a mixer into the cuvette. The spectrofluorometer delivered light at 410 nm, which passed through the cuvette, and the intensity of transmittance was detected at the other end.

The other electronics associated with the spectrofluorometer and stopped flow instruments were arranged in the bottom of the cart. During the microgravity experiments, these were held in place by cargo straps hooking to U bolts on the walls of the cart. The second cart was used to hold the computer's monitor, trackball mouse, keyboard and a Thermotek solid-state water bath. For the microgravity experiments,

these were attached using industrial strength Velcro and the monitor and water bath were additionally attached using multiple cargo straps.

During the experiments, the progress of the AP-pNPP reaction was monitored by the change in transmittance through the cuvette at 410 nm for 10 seconds. The raw data obtained from these experiments are plots of transmittance intensity versus time. These experiments were done over a range of pNPP concentrations from 0.1 mM to 1.5 mM while the AP concentration stayed constant at 0.4 mg/ml. The initial linear portion of the trace corresponding to each pNPP concentration was fit to a line and the slope was determined. This slope was defined as the initial velocity ($V_{initial}$) at that particular pNPP concentration and the unit of $V_{initial}$ was relative transmittance units/millisecond/milligram of enzyme (RTU ms^{-1} mg^{-1}). The relative transmittance units could not be translated into amount of product because there was no concentration standard for the product.

The $V_{initial}$s were plotted against the pNPP concentrations to get a hyperbolic plot. This plot could be fit to the Michaelis Menten equation using the graphing software ,Kaleidagraph, and values for the kinetic parameters, K_m and V_{max} were obtained.

Figure D: Cart 1 with the ISS Koala spectrofluorometer on the top of the cart and a Bio-Logic SFM 3 stopped-flow in the center. The other equipment associated with these instruments are in the bottom of the cart

Figure E: Cart 2 holding the computer monitor, keyboard and trackball mouse. The water bath is placed in the bottom of the cart

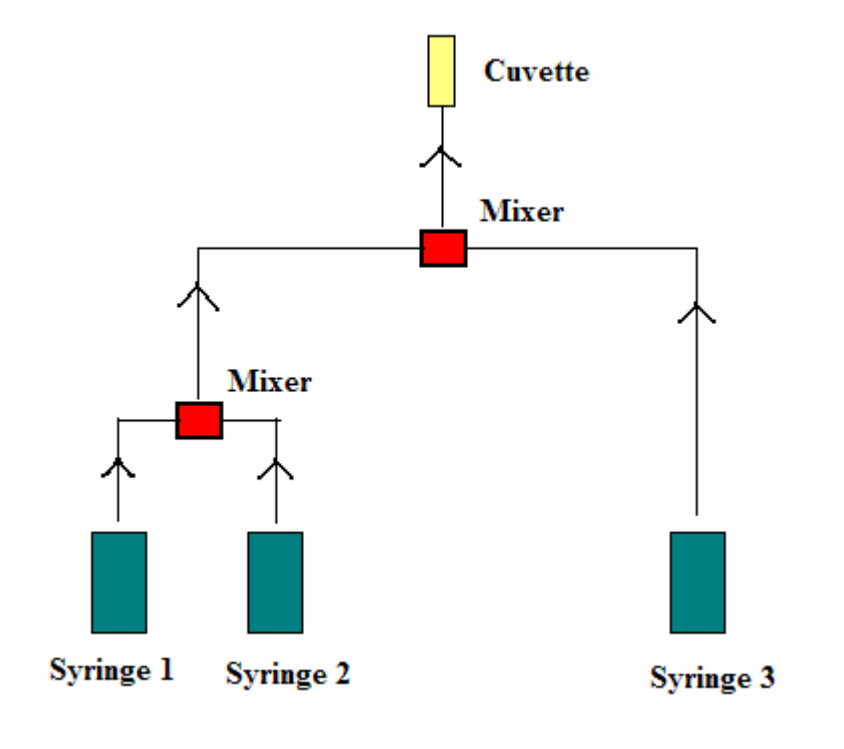


Figure F: Basic set-up of stopped flow used for AP experiments in this study

RESULTS

I. Reaction conditions

The reaction took place in 0.6 M Tris buffer, pH 8.2 and at 25°C. Figure 1 shows an example of some traces. The portion of the traces corresponding to the initial 0.6 seconds was linearly fit to obtain the $V_{initial}$. Figure 2 shows the plot of these $V_{initial}$s against the concentrations of pNPP and the fit to the Michaelis Menten equation to get a V_{max} of 78.7 RTU ms^{-1} mg^{-1} and a K_m of 0.032 mM.

Different reaction conditions were tested in an effort to slow the reaction down. This was done in order to be able to fit the reaction to the microgravity time window. It would also lengthen the linear portion of the trace and allow a better linear fit for $V_{initial}$. Changing the pH of the buffer to 10 did not affect the reaction much. It decreased the $V_{initial}$ slightly. The linear portion of the trace was also extended slightly to 0.7 seconds. The Michelis Menten plot at this pH is shown in Figure 3. The V_{max} obtained was 73.1 RTU ms^{-1} $mg^{-1}ms^{-1}$, which was not considerably different from the V_{max} for the reaction at pH 8.2 but the K_m was 0.069 mM, which is nearly twice that at the lower pH.

The next experiments were conducted in 0.6 M Tris, pH 8.2 and at 12°C. The reaction was considerably slowed down at this temperature. There was at least a 60% decrease in the $V_{initial}$ for the various pNPP concentrations at this temperature. The length of the linear portion was also extended to 1.5 seconds. Figure 4 compares two traces at the same pNPP concentration at 25°C and 12°C. It clearly shows the slowing down of the reaction at lower temperature. The Michaelis Menten plot for the experiments at 12°C is shown in Figure 5. There was a 60% decrease in the V_{max} to 31.6 RTU ms^{-1} mg^{-1} and a 45% increase in the K_m to 0.047 mM compared to the reaction at 25°C.

A change in the Tris concentration of the buffer from 0.6 M to 0.01 M also caused the reaction to slow down considerably. The next trials were carried out at 12°C and in 0.01 M Tris buffer, pH 8.2. The $V_{initial}$s decreased by nearly 90% under these conditions. The initial 4 seconds of the trace could be fit to a line for these $V_{initial}$s. A comparison of traces at the same pNPP concentration in these conditions and in 0.6 M Tris, pH 8.2 at 25°C is shown in Figure 6. The K_m and V_{max} obtained from the Michaelis Menten plot (Figure 7) are 0.025 mM and 8.13 RTU ms^{-1} mg^{-1}. This shows a 90% decrease in the V_{max} and a 20% decrease in the K_m at these conditions compared to the experiments in 0.6 M Tris buffer at 25°C.

Reaction conditions of 0.01 M Tris, pH 8.2 buffer at 12°C were used for all subsequent experiments because the slowest $V_{initial}$s were observed in these conditions and the linear portion was also extended up to 4 seconds for the best fit for $V_{initial}$.

II. Viscosity experiments

Once the conditions for a slower reaction were determined, the viscosity experiments were conducted to investigate the extent of diffusion control for the reaction. The experiments were conducted in buffers with 0%, 10%, 20% and 30% (v/v) glycerol. The viscosity of the buffer, η, was determined using a viscometer. The $V_{initial}$s for these experiments were obtained by a linear fit to the initial 4 seconds of the trace. There was a decrease in $V_{initial}$ at any particular pNPP concentration with increasing viscosity. Figure 8 shows the Michaelis Menten plot for these viscosity experiments. The kinetic parameters from these experiments are listed in Table 1. The V_{max} clearly decreases with increasing viscosity and there is only a slight increase in the K_m values. This translates into an increase in V_{max}/K_m with increasing viscosity.

In order to determine the relationship between the V_{max}/K_m and the viscosity, the relative V_{max}/K_m ($(V_{max}/K_m)°/(V_{max}/K_m)$) and the relative viscosity ($\eta/\eta°$) were calculated and plotted. The superscript ° here denotes the reference state. According to the Einstein-Stokes equation, diffusion is inversely proportional to the viscosity of the solution.

$$D = \frac{kT}{6\pi r\mu}$$

where D = Brownian diffusivity
k = Boltzman's constant
T = absolute temperature (K)
r = radius of the molecule
μ = viscosity of the medium

This implies the rate of a diffusion-controlled reaction is also inversely proportional to the viscosity. Therefore, a plot of the relative V_{max}/K_m versus relative viscosity should show a slope of 1.00 for a reaction that is 100% diffusion-controlled. Figure 9 shows this plot of $(V_{max}/K_m)°/(V_{max}/K_m)$ versus $\eta/\eta°$. The slope obtained, 1.2992 is bigger than 1 but it indicates that the AP-pNPP reaction is diffusion controlled. The plots of relative Km (Km/Km°) versus $\eta/\eta°$ and relative Vmax (Vmax°/Vmax) versus $\eta/\eta°$ are also linear with slopes 0.3026 and 0.6058 (Figure 9).

III. Microgravity experiments

The effect of gravity on the kinetics of the diffusion controlled AP-pNPP reaction was investigated. The viscosity experiments were repeated in conditions of 0 G and 1.8 G, that is, in the absence of gravity and under gravity 1.8 times that of normal gravity. Experimental conditions in the C9 airplane are considerably harsher than in the lab. This significantly increased the noise level in the data. Thus, many of the traces obtained at 0 G and 1.8 G required excisions of some portions of the trace in order to do a linear fit for

$V_{initial}$ (Figure 10). The whole trace was considered for the linear fit, not just the initial 4 seconds. Some of the traces at 0 G were multiphasic and could be fit to more than one line (Figure 10). The $V_{initial}$ in these cases were reasonably chosen based on the range of values expected for the $V_{initial}$ at that pNPP concentration.

Figures 11, 12, 13 and 14 show the Michaelis Menten plots for the reaction in different gravitational conditions in buffers containing 0%, 10%, 20% and 30% glycerol, respectively. The kinetic parameters obtained from the plot are tabulated in Table 2. The data for the reaction at 1.8 G in 0% glycerol buffer could not be fit to the Michaelis-Menten equation. The K_m and V_{max} values obtained for the 1 G experiments shown in this table are different from the previous viscosity data. However, the origin of this difference could not be determined.

There does not appear to be a definitive pattern of change in the K_m and V_{max} values as a result of these different gravitational conditions. The relative V_{max}/K_m versus $\eta/\eta°$ was also plotted for 0 G and 1 G (Figure 15). The sloped obtained were 0.6497 fro 0 G and 0.2520 for 1 G but the linear fit for the data was not very good as illustrated by the R^2 values of 75% and 60% respectively. The fits for relative K_m and relative V_{max} versus $\eta/\eta°$ for 0 G and 1 G are also shown in Figure 15. The R^2 values for these linear fits were even lower. Similar plots could not be constructed for 1.8 G because there was no 0% glycerol data that could be used as reference. The absence of a pattern of change in K_m and Vmax with changing gravitational conditions and the poor linear fits shown in Figure 15 lead us to conclude that a change in gravity seems has no effect on this reaction.

DISCUSSION:

I. Reaction conditions

The effect of the change in the variables such as temperature, pH and Tris concentration on the kinetic parameters of the AP-pNPP reaction can be explained by observing the role of these variables in the reaction.

Changing the pH from 8.2 to 10 did not produce a considerable change in the $V_{initial}$ of the reaction. It may be that the change in pH was not big enough to cause a major effect on the reaction velocity. The increase in K_m at pH 10 relative to that at pH 8.2 and the similarity in V_{max} at pH 8.2 and 10 have been observed in studies by Krishnaswamy and Kenkare as well [9]. This indicates that the protonation of a lysine or arginine is optimal for substrate binding. But the increased pH did not slow the reaction as desired so subsequent experiments were conducted at pH 8.2.

Decrease in temperature causes a drop in the rate of all reactions in accordance with the Arrhenius equation, $k=Ae^{-\Delta G^*/RT}$ where k is the kinetic rate constant for the reaction, A is the Arrhenius constant, also known as the frequency factor, ΔG^* is the standard free energy of activation, R is the gas law constant and T is the absolute temperature. At a lower temperature, molecular motion slows down and this causes reduced interaction between molecules. Fewer interaction lead to reduced rates of reaction. Therefore, the change in temperature from 25°C to 12°C causes a decrease in the $V_{initial}$. The decrease in V_{max} and increase in K_m also reflects this slower reaction.

A decrease in Tris concentration slows the AP-pNPP reaction. This can be understood by examining the role of Tris in the reaction. The dephosphorylation of the phosphoryl enzyme intermediate to release free enzyme is enhanced in the presence of

Tris which is a good phosphate acceptor (k3', k4' – Scheme 1). In other words, Tris leads to the dephosphorylation of the phosphoryl AP enzyme intermediate by favoring the formation of a Tris-phosphate complex. At pH less than 7, hydrolysis of the phosphoenzyme (k3) is the rate limiting step. At pH greater than 7, phosphoenzyme hydrolysis exceeds formation of the phosphoenzyme but the dissociation of Pi (k4) is rate limiting [10, 11]. Since the pH used for the experiments is 8.2, the rate limiting step is dissociation of Pi. Therefore at lower concentrations of Tris, the phosphoryl enzyme is converted into free enzyme mostly by the dissociation of the inorganic phosphate from the enzyme and this occurs much more slowly. Thus decreased Tris concentrations leads to slower availability of free AP enzyme to catalyze the reaction and slows down the reaction as a whole.

II. Viscosity experiments

The dependence of this reaction on viscosity was examined to determine the extent of diffusion control of the reaction. The glycerol experiments showed a decrease in V_{max}/K_m with increasing viscosity. Simopoulos and Jencks also report a large decrease in the k_{cat}/K_m for the AP-pNPP reaction at pH 8 with increasing glycerol concentrations. Since k_{cat} is directly proportional to the V_{max}, their results are in agreement with the results reported here. Their plot of relative k_{cat}/K_m ($(k_{cat}/K_m)°/(k_{cat}/K_m)$) versus relative viscosity ($\eta/\eta°$) yields a slope of 4. This is much larger than the slope expected for a diffusion controlled reaction. Simopoulos and Jencks propose that in addition to an effect on viscosity, glycerol reduces the rate of the reaction by binding to the active site of the enzyme or by a solvent effect which decreases the catalytic activity of the enzyme. They support this by the results from an experiment done at pH 6. High concentrations of

glycerol reduced the first order rate constant for the hydrolysis of phosphoenzyme intermediate, which is the rate limiting step for the AP-pNPP reaction at pH < 7 and this indicates a solvent effect by glycerol on the conformation of the enzyme that affects its catalytic activity [1].

Simopoulos and Jencks report that similar experiments with the first order rate constant at pH 6 done with sucrose showed no effects. Thus, sucrose does not affect the structure of the enzyme that affects catalytic activity. A plot of relative kcat/K_m versus relative viscosity for sucrose viscosity experiments showed a slope of 1.4. The authors state that these results along with results from experiments with other viscogens indicate that the AP-pNPP reaction is diffusion controlled [1].

Although the plot of relative V_{max}/K_m versus relative viscosity for our glycerol experiments did not produce a slope as large as 4, it was still higher than 1 indicating diffusion control. The difference in slopes between our experiments and the experiments conducted by Simpoulos and Jencks may arise due to differences in the reaction conditions used. They conducted their experiments at a temperature of 25°C and the buffer used for their experiments also had higher concentrations of Tris. The authors do not report the source organism for the alkaline phosphatase used for their experiments. If they did not used *E. coli* AP, this may also explain the difference in the slopes. Our slope of 1.2992 from glycerol viscosity experiments is also very similar to the slope of 1.4 that they obtained with sucrose experiments. Therefore, the results of Simpoulos and Jencks support our findings.

III. Microgravity experiments

The AP-pNPP reaction which is believed to be diffusion-controlled was examined in conditions of zero gravity and 1.8 times gravity. The results showed that the kinetic parameters V_{max} and K_m did not change in a recognizable pattern from 0 G to 1 G to 1.8 G at all viscosities. The plots of relative V_{max}/K_m, relative V_{max} and relative K_m versus relative viscosity at 0 G and 1 G also did not produce good linear fits. Thus increase in gravity or the absence of gravity seems to have no effect on the kinetics of the enzymatic reaction. This agrees with the results of microgravity experiments with isocitrate lyase. The study by Giachetti *et al* [12, 13] showed that the isocitrate lyase reaction obeyed the same kinetic mechanism at microgravity and none of the kinetic parameters were altered. But studies with lipoxygenase-1 show that the rate of reaction is enhanced at low gravity [14]. Therefore, it is possible that some reactions are sensitive to the influence of gravity while others are not. However, the data collected for the microgravity experiments with alkaline phosphatase is insufficient to claim that gravity influences the rate of this reaction. Future experiments with more data points should provide more definitive results.

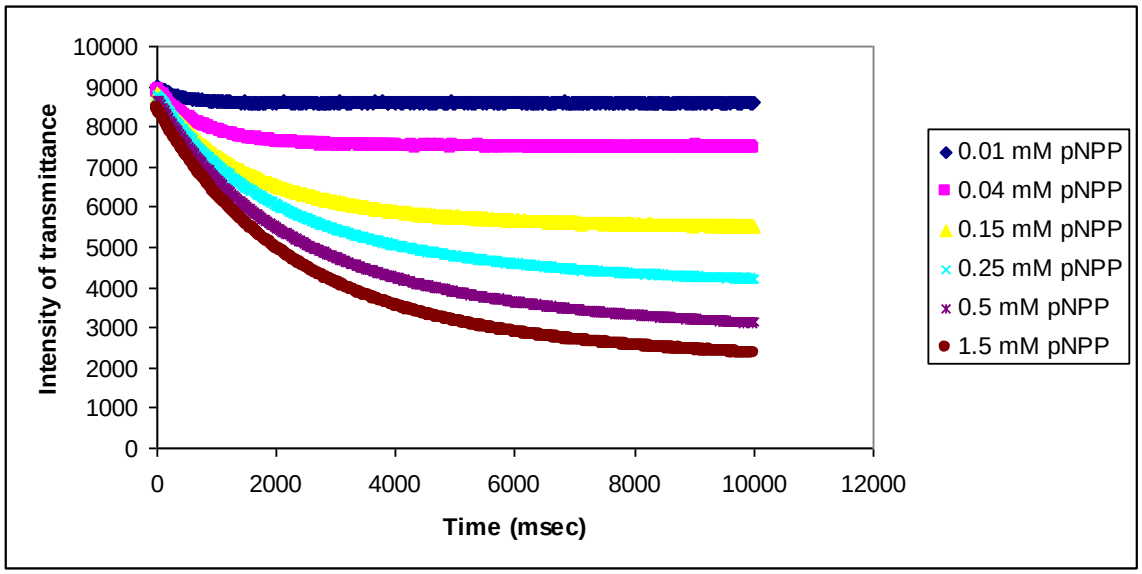


Figure 1: Traces obtained at different pNPP concentrations are shown. Each trace shows the AP-pNPP reaction as the change in transmittance over 10 seconds for that particular pNPP concentration.

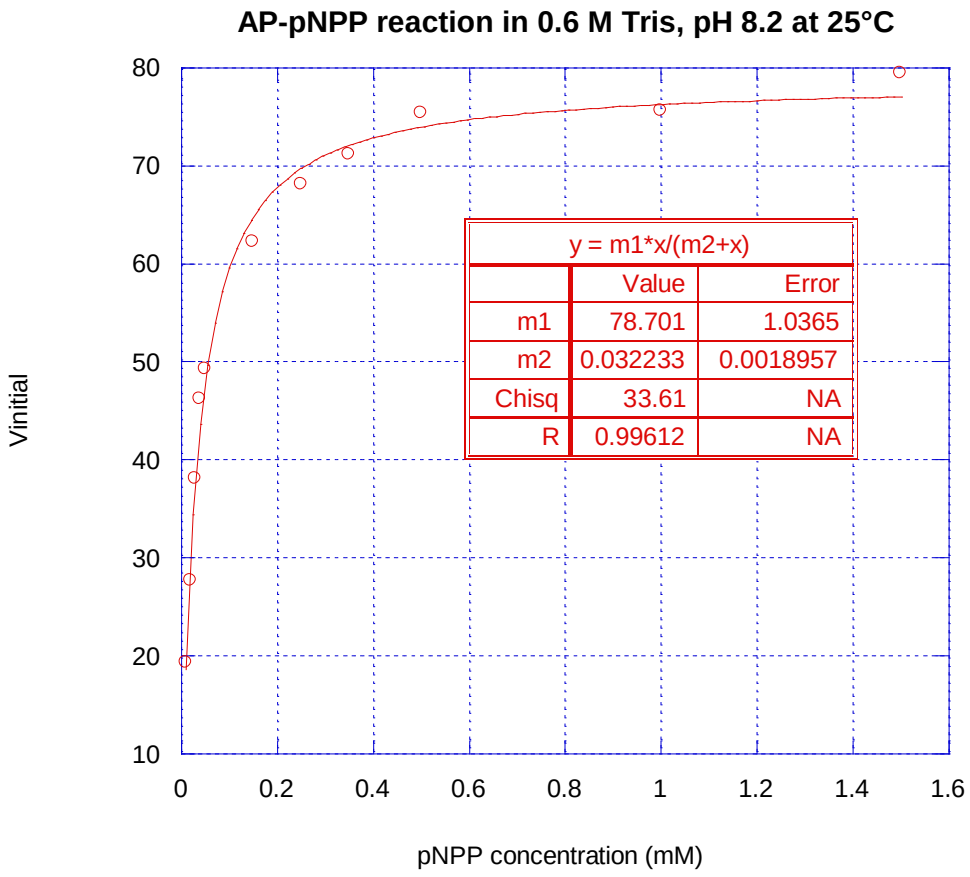


Figure 2: Michaelis-Menten plot of $V_{initial}$ vs pNPP concentration for the AP-pNPP reaction in 0.6 M Tris, pH 8.2 at 25°C

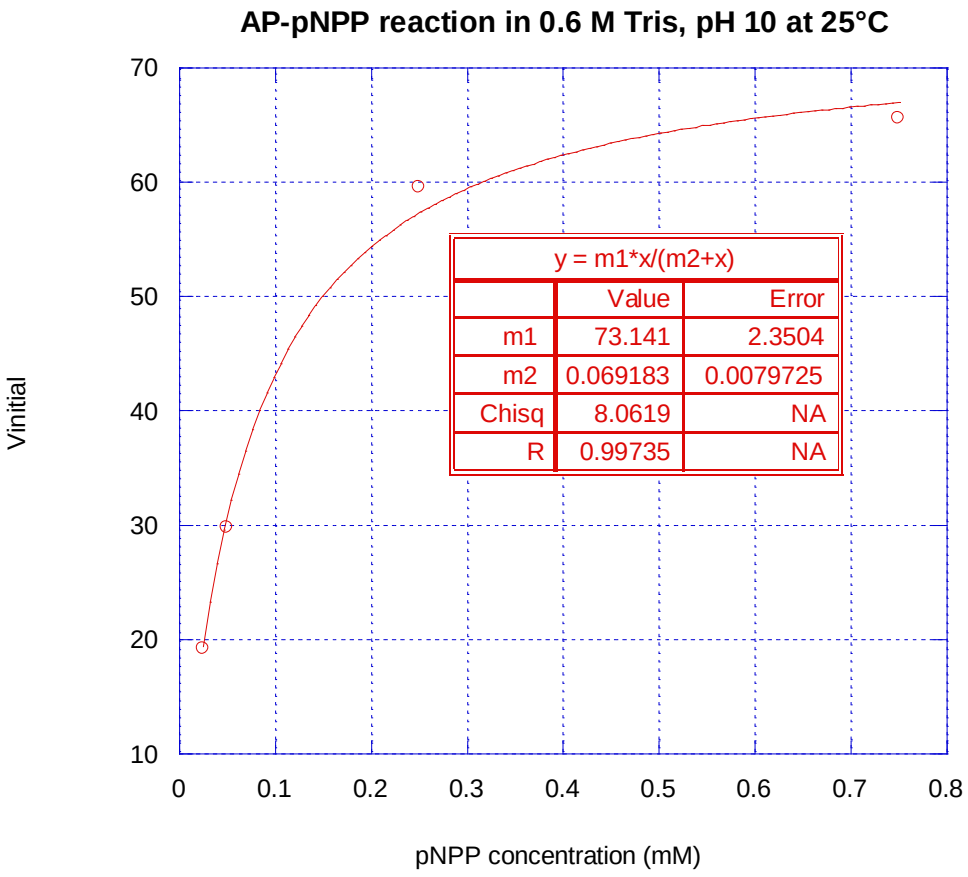


Figure 3: Michaelis-Menten plot of $V_{initial}$ vs pNPP concentration for the AP-pNPP reaction in 0.6 M Tris, pH 10 at 25°C

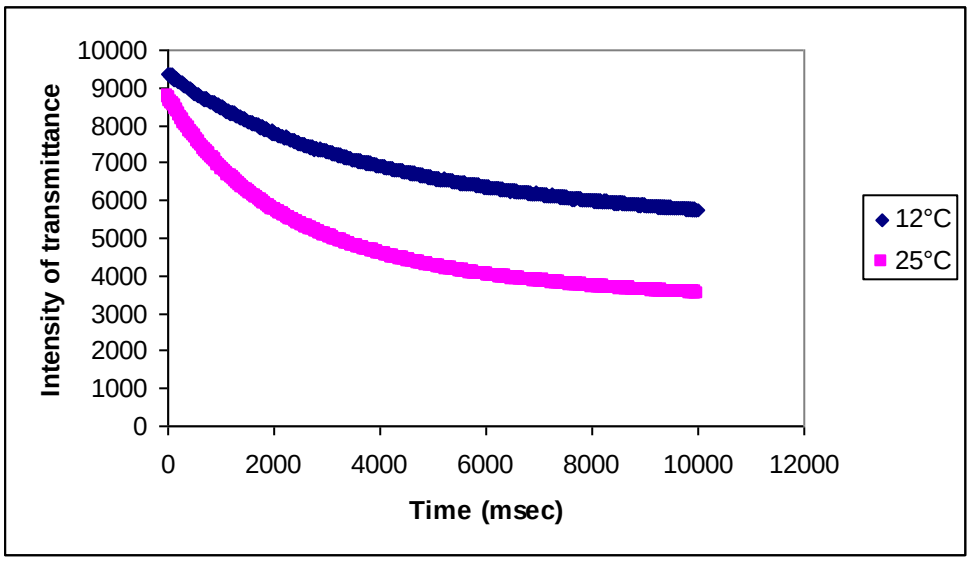


Figure 4: Traces of AP-pNPP reaction at the same pNPP concentration but at different temperatures, 25°C and 12°C. These traces show that the reaction is slower at 12°C compared to 25°C

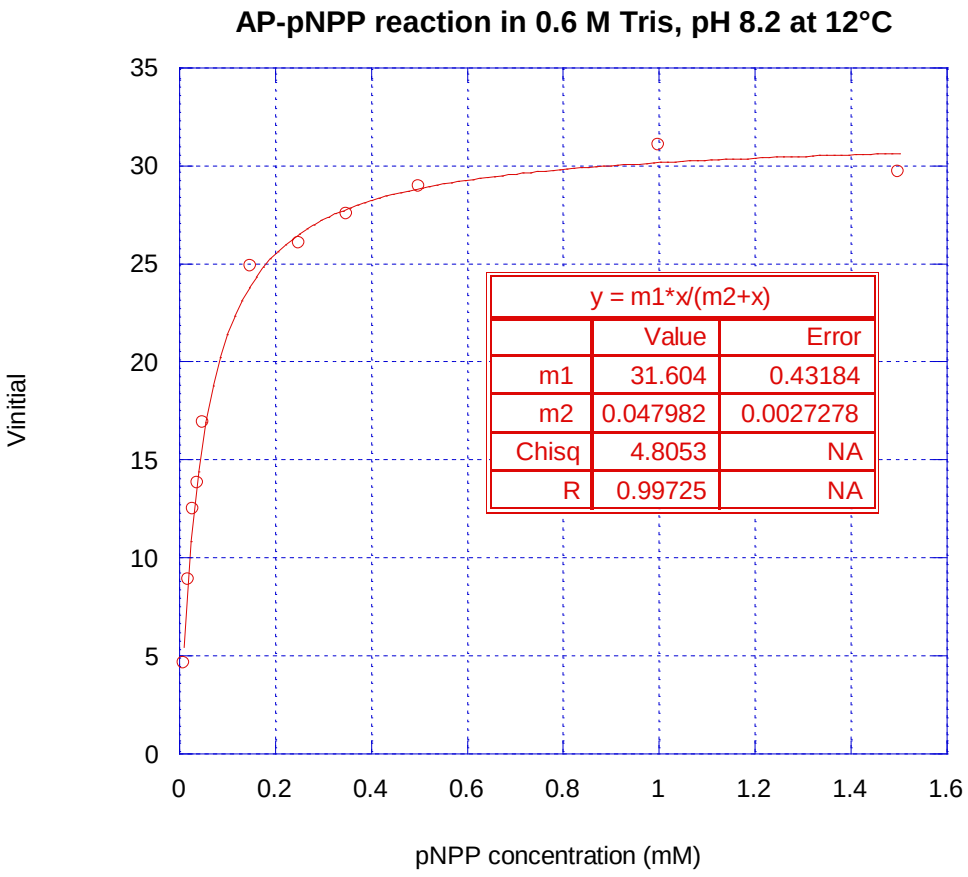


Figure 5: Michaelis-Menten plot of $V_{initial}$ vs pNPP concentration for the AP-pNPP reaction in 0.6 M Tris, pH 8.2 at 12°C

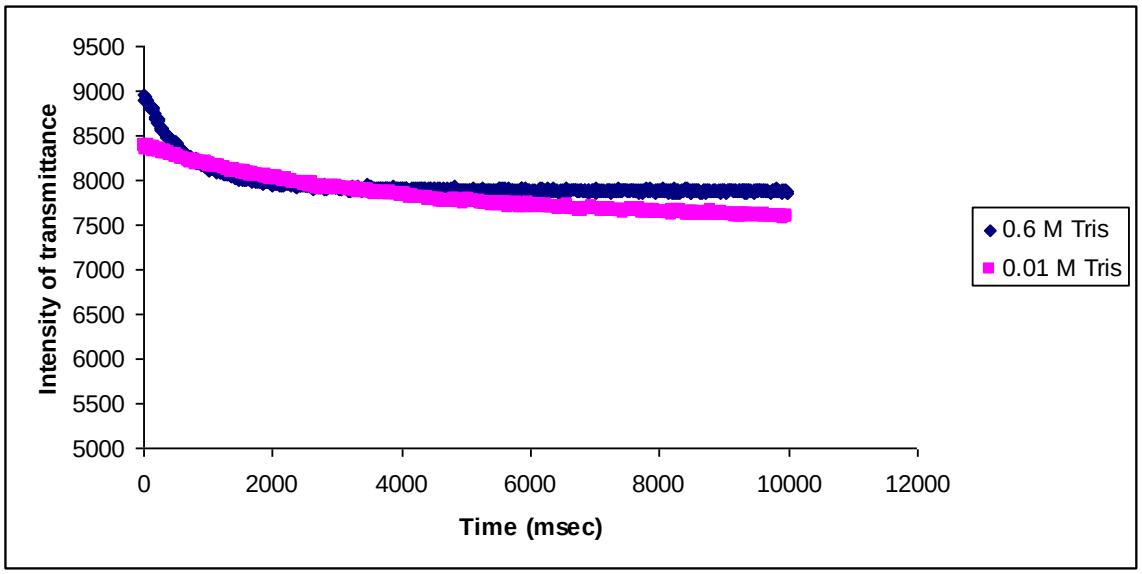


Figure 6: Traces of AP-pNPP reaction at the same pNPP concentration but in different buffers, 0.6 M Tris and 0.01 M Tris. These traces show that the reaction is slower in 0.01 M Tris buffer compared to 0.6 M Tris.

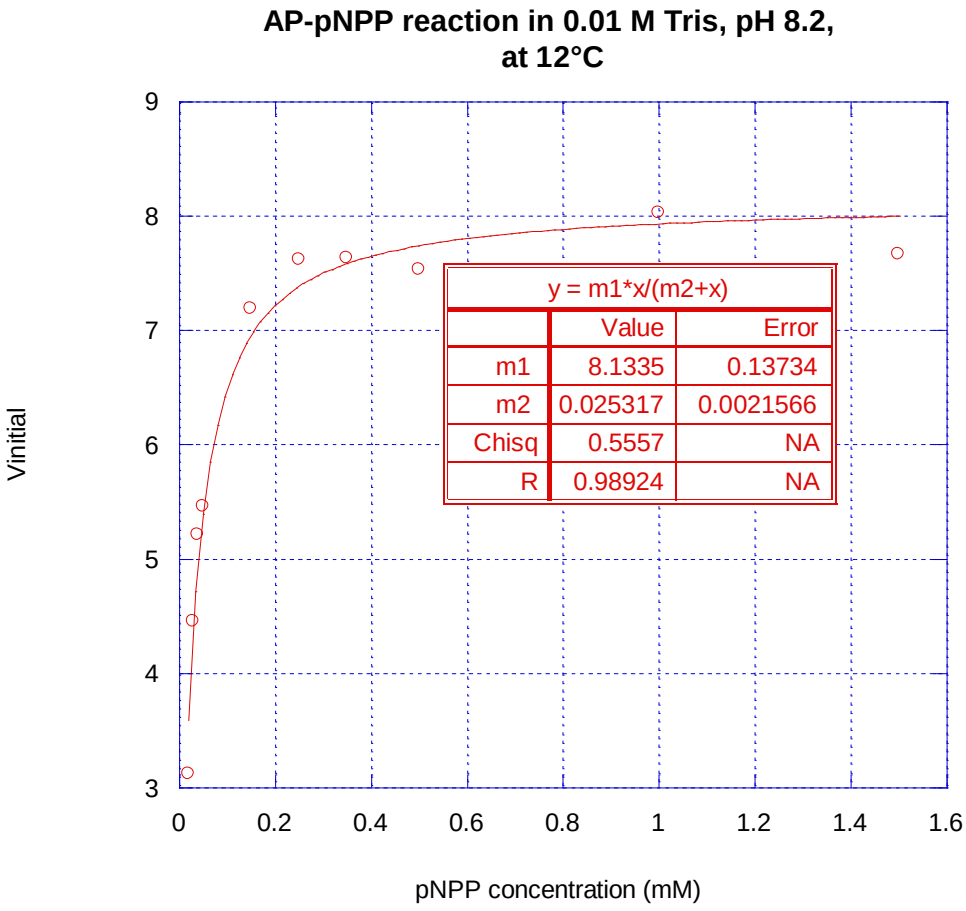


Figure 7: Michaelis-Menten plot of $V_{initial}$ vs pNPP concentration for the AP-pNPP reaction in 0.01 M Tris, pH 8.2 at 12°C

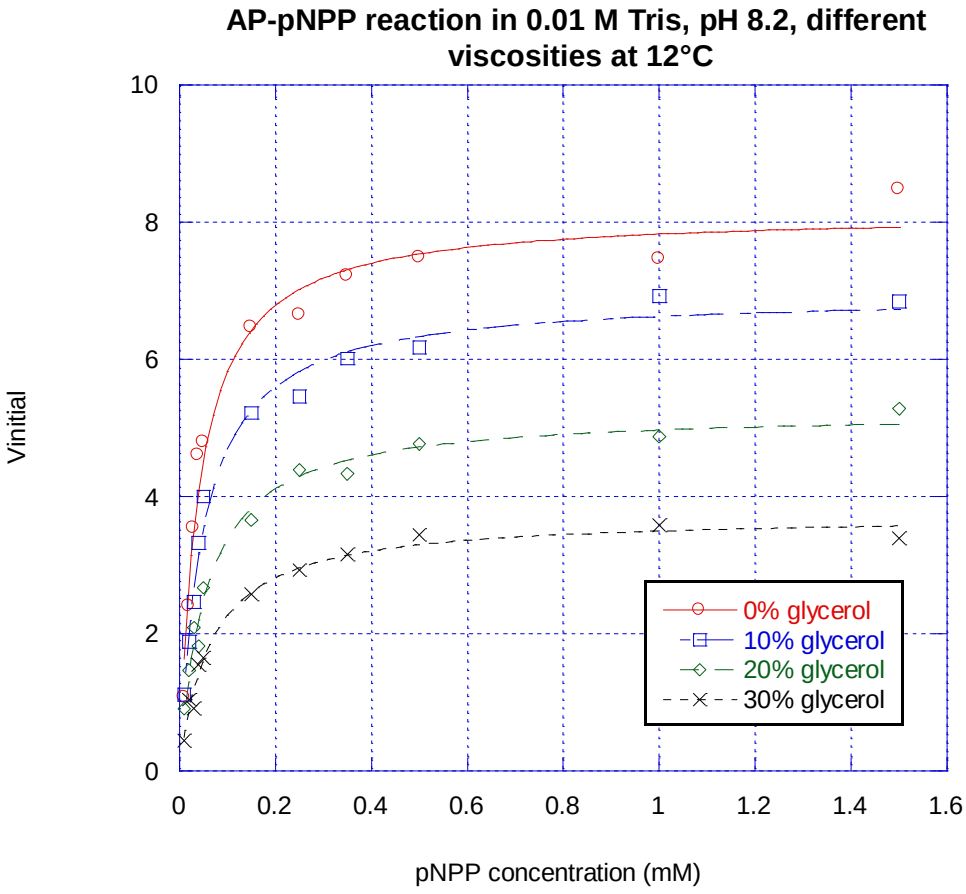
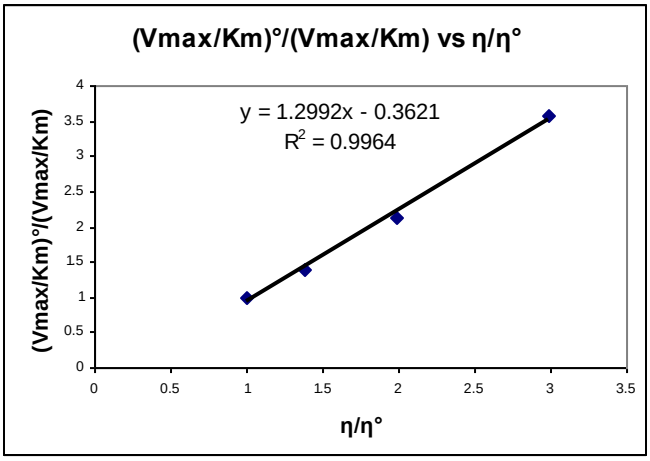
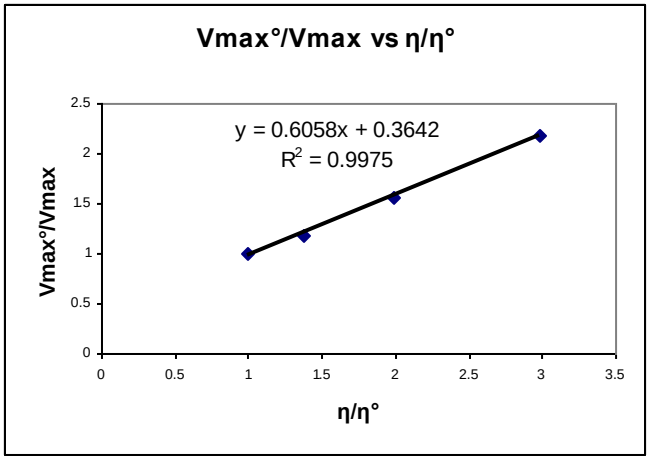


Figure 8: Michaelis-Menten plot of $V_{initial}$ vs pNPP concentration for the AP-pNPP reaction in 0%, 10%, 20% and 30% glycerol buffers.





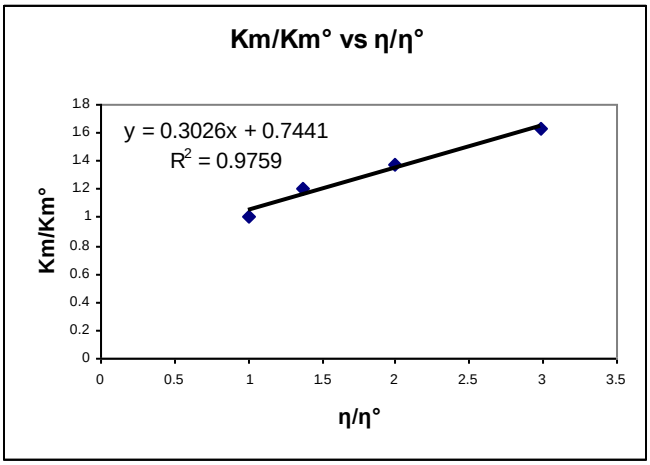
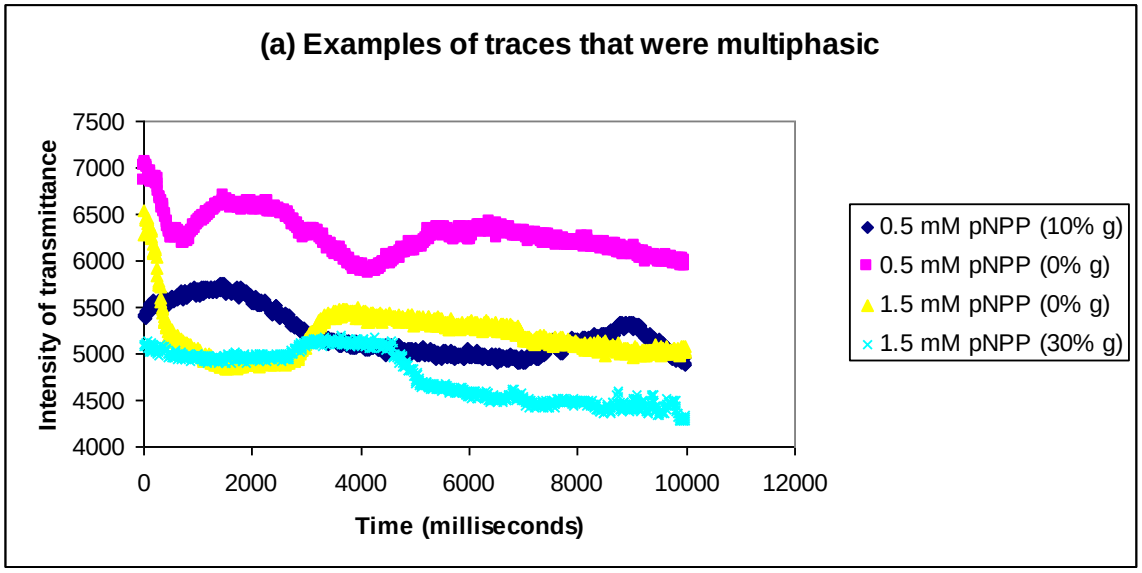


Figure 9: Plot of relative V_{max}/K_m, relative V_{max} and relative K_m versus relative viscosity



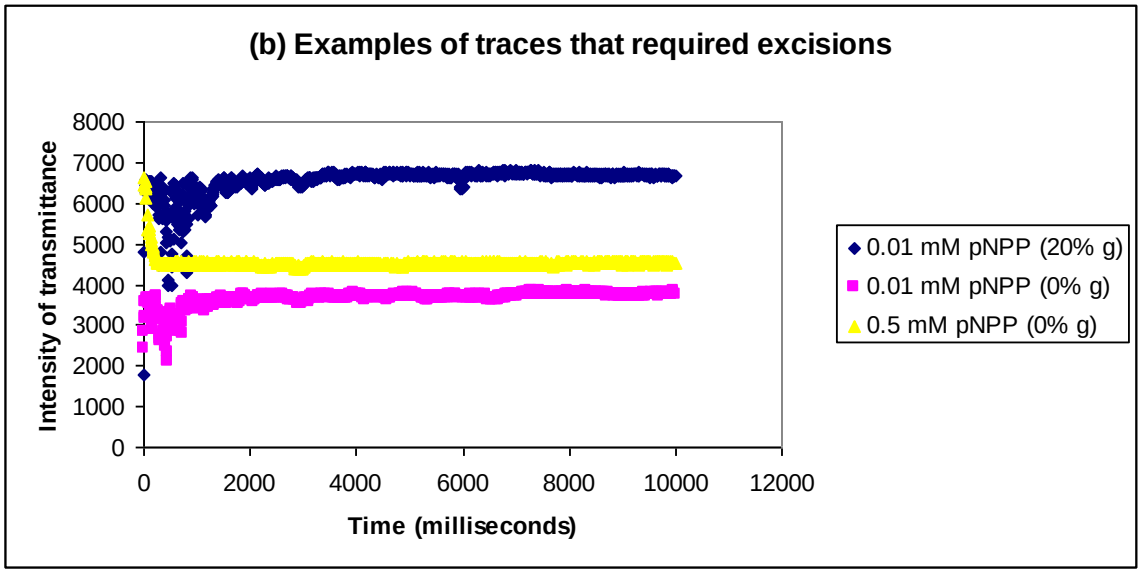


Figure 10: (a) Traces that were multiphasic and could be fit to more than one line for $V_{initial}$. The examples shown here correspond to reactions at 0 G in buffers containing 0%, 10% and 30% glycerol (b) Traces that required excisions to fit for $V_{initial}$. The examples shown here correspond to reactions at 2 G in buffers containing 0% and 20% glycerol.

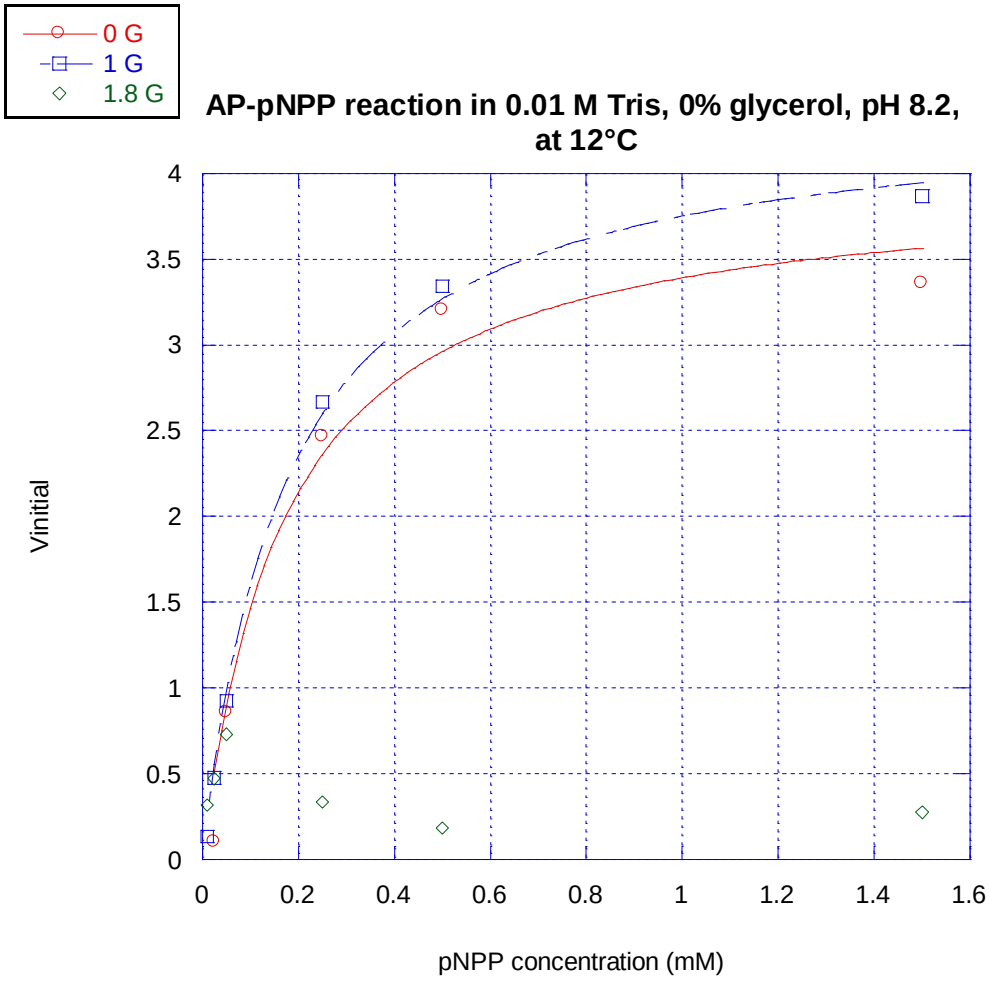


Figure 11: Michaelis-Menten plot of $V_{initial}$ vs pNPP concentration for the AP-pNPP reaction in 0% glycerol buffer at gravitational conditions 0 G, 1 G and 1.8 G

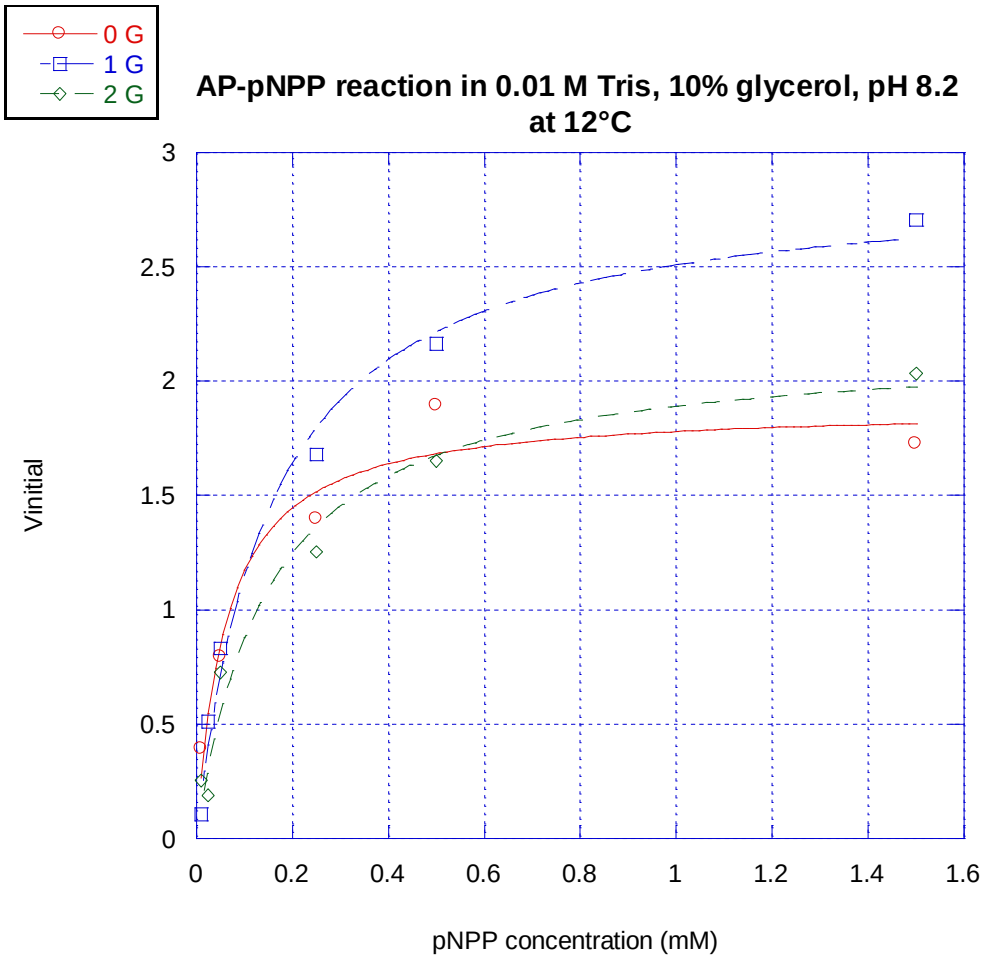


Figure 12: Michaelis-Menten plot of $V_{initial}$ vs pNPP concentration for the AP-pNPP reaction in 10% glycerol buffer at gravitational conditions 0 G, 1 G and 1.8 G

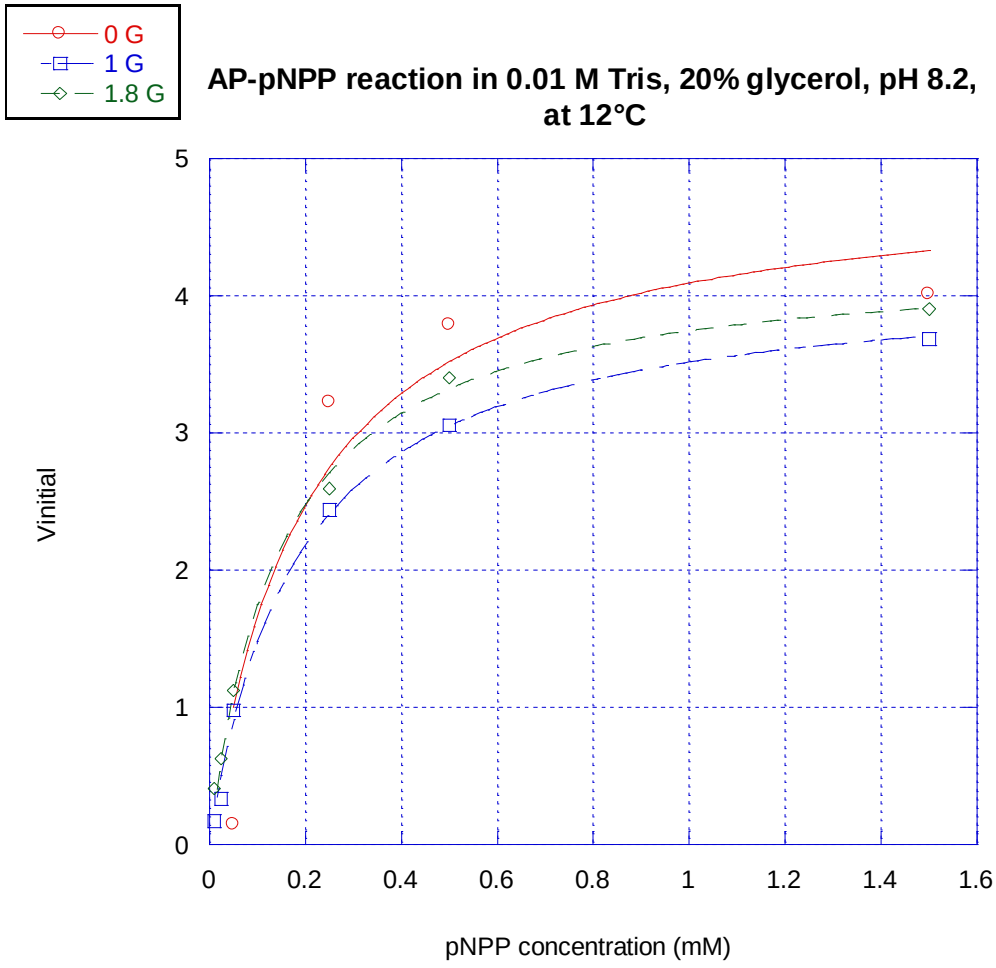


Figure 13: Michaelis-Menten plot of $V_{initial}$ vs pNPP concentration for the AP-pNPP reaction in 20% glycerol buffer at gravitational conditions 0 G, 1 G and 1.8 G

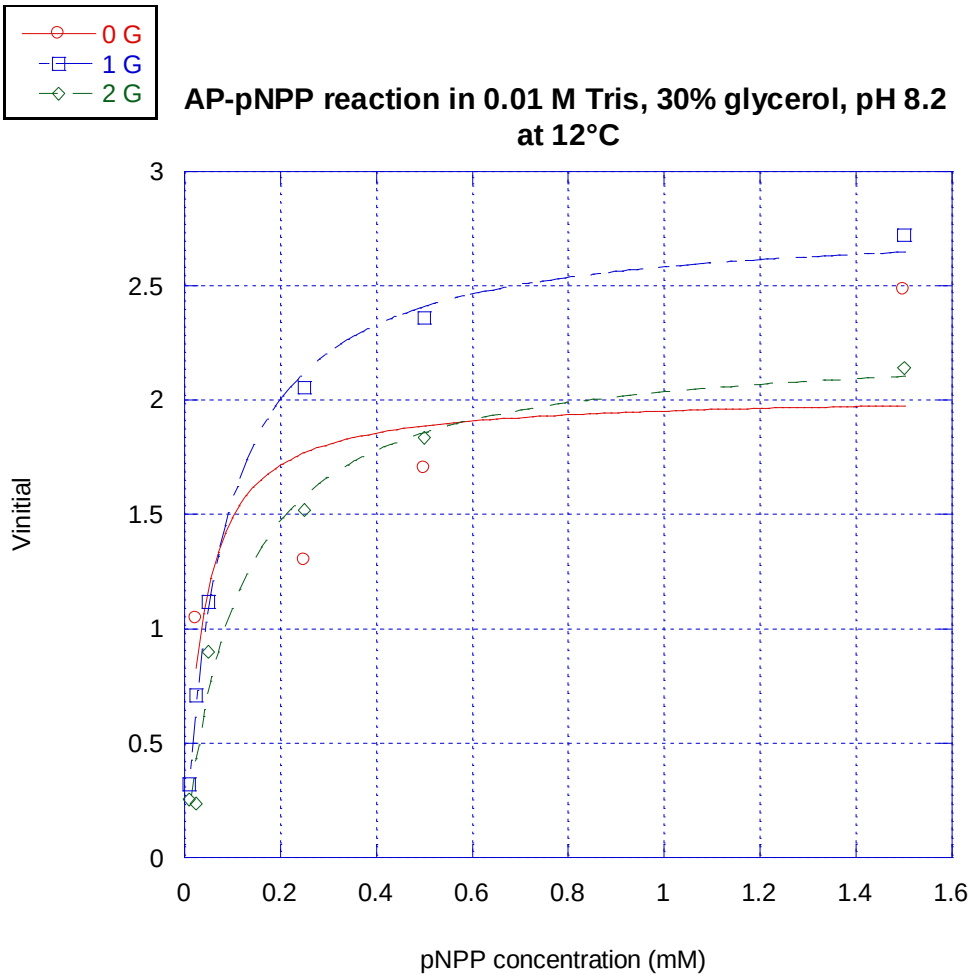


Figure 14: Michaelis-Menten plot of $V_{initial}$ vs pNPP concentration for the AP-pNPP reaction in 30% glycerol buffer at gravitational conditions 0 G, 1 G and 1.8 G

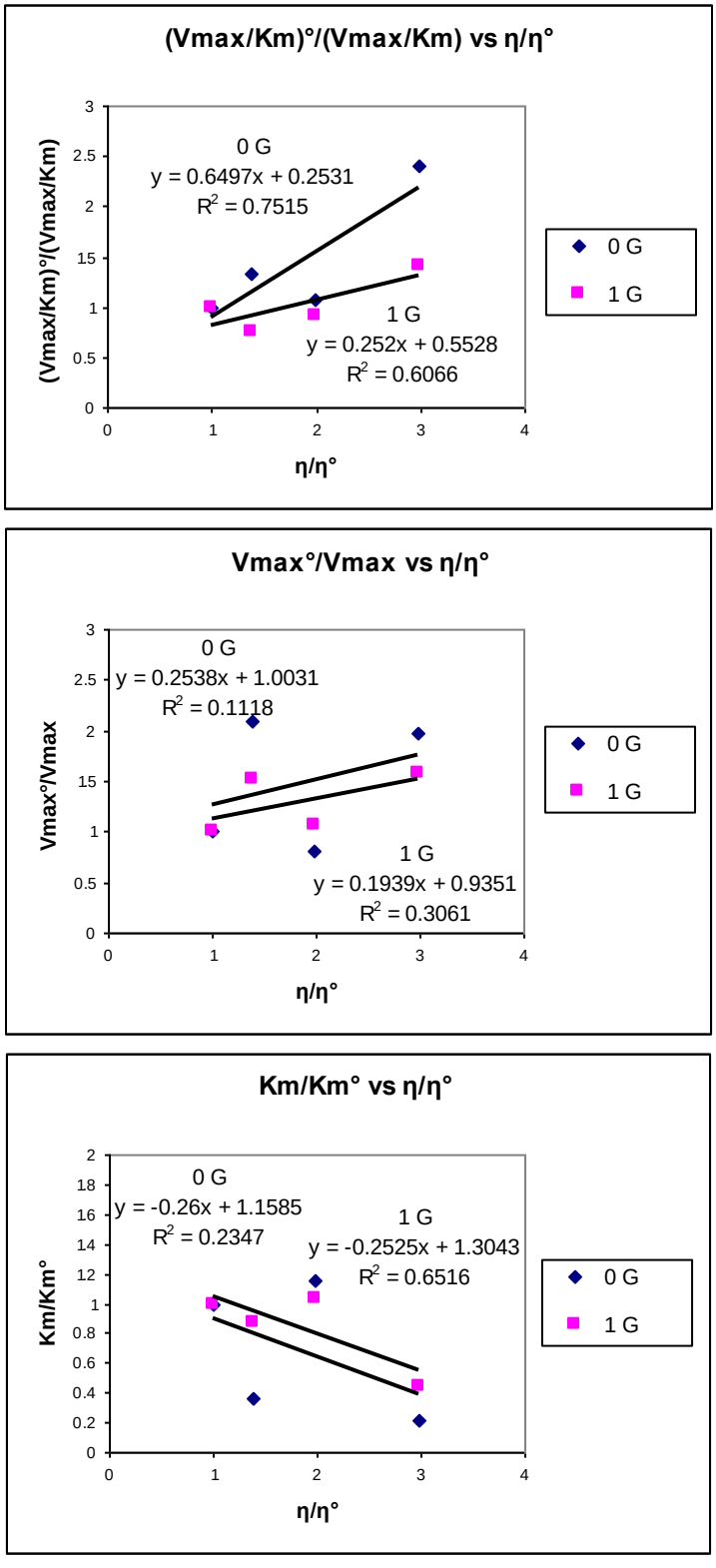


Figure 15: Plot of relative V_{max}/K_m, relative V_{max} and relative K_m vs η/η° at 0 G and 1 G

	K_m(mM)	V_{max} (RTU $ms^{-1}mg^{-1}$)	V_{max} / K_m
0% glycerol	0.040 ± 0.005	8.13 ± 0.22	203 ± 44
10% glycerol	0.048 ± 0.004	6.94 ± 0.15	145 ± 38
20% glycerol	0.055 ± 0.006	5.24 ± 0.13	95 ± 22
30% glycerol	0.065 ± 0.006	3.72 ± 0.09	57 ± 15

Table 1: The kinetic parameters obtained from the Michaelis Menten plots from Figure 8 for the viscosity experiments are tabulated here

	K_m(mM)	V_{max} (RTU $ms^{-1}mg^{-1}$)	V_{max} / K_m
0% glycerol			
0 G	0.170 ± 0.061	3.97 ± 0.40	23.4 ± 6.6
1 G	0.174 ± 0.018	4.40 ± 0.13	25.3 ± 7.2
1.8 G	----------	----------	---------
10% glycerol			
0 G	0.061 ± 0.023	1.89 ± 0.15	31.0 ± 6.5
1 G	0.152 ± 0.027	2.89 ± 0.14	19.0 ± 5.2
1.8 G	0.148 ± 0.042	2.17 ± 0.17	14.7 ± 4.0
20% glycerol			
0 G	0.196 ± 0.153	4.89 ± 1.11	24.9 ± 7.3
1 G	0.181 ± 0.020	4.15 ± 0.13	22.9 ± 6.5
1.8 G	0.146 ± 0.015	4.29 ± 0.12	29.4 ± 8.0
30% glycerol			
0 G	0.036 ± 0.043	2.02 ± 0.40	56.1 ± 9.3
1 G	0.078 ± 0.006	2.78 ± 0.06	35.6 ± 10.0
1.8 G	0.106 ± 0.027	2.25 ± 0.14	21.2 ± 5.2

Table 2: The kinetic parameters from the Michaelis Menten plots from Figures 11-14 are tabulated here

REFERENCES:

1. Simpoulos, T. T. and Jencks, W. P. (1994). Alkaline phosphatase is an almost perfect enzyme. *Biochemistry* 33: 10375-80
2. Coleman, J. E. (1992). Structure and mechanism of alkaline phosphatase. *Annual Review of Biophysics and Biomolecular structure* 21: 441-83
3. Berg, J. M., Tymoczko, J. L., Stryer, L. (2002). Biochemistry
4. Gekko, K. and Timasheff, S. N. (1981). Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry* 20(16): 4667-76
5. Fleming, O. S., Reddy, S., Mantalaris, A. (2002). Advances in the study of bone development in simulated microgravity. *European Cells and Materials* 4(S2): 123-124
6. Mornet, E., Stura, E., Lia-Baldini, A., Stigbrand, T., Menez, A., Le Du, M. (2001). Structural evidence for a functional role of human tissue nonspecific alklaine phosphatase in bone mineralization. *The Journal of Biological Chemistry* 276(33): 31171-78
7. Information sheet from Canadian Space Agency: Space travel and the effects of weightlessness on the human body
8. Zerath, E., Novikov, V., Leblanc, A., Bakulin, A., Oganov, V., Grynpas, M. (1996). Effect of spaceflight on bone mineralization in the rhesus monkey. *Journal of applied physiology* 81(1): 194-200

9. Krishnaswamy, M. and Kenkare, U. W. (1970). The effect of pH, temperature, and organic solvents on the kinetic parameters of *Escherichia coli* alkaline phosphatase. *The Journal of Biological Chemistry* 245(15): 3956-63
10. Zhang, L., Buchet, R., Azzar, G. (2004). Phosphate binding in the active site of alkaline phosphatase and the interactions of 2-nitrosoacetophenone with alkaline phosphatase-induced small structural changes. *Biophysical Journal* 86: 3873-81
11. Trentham, D. R. and Gufreund, H. (1968). The kinetics of the reaction of nitrophenyl phosphates with alkaline phosphatase from *Escherichia coli*. *The Biochemical Journal* 106(2): 455-60
12. Giachetti, E., Ranaldi, F., Vanni, P. (2001). Enzyme catalysis in microgravity: An intricate problem to be solved. *FEBS Letters* 504: 78-79
13. Ranaldi, F., Vanni, P., Giachetti, E. (2003). Enzyme catalysis in microgravity: steady-state kinetic analysis of the isocitrate lyase reaction. *Biophysical Chemistry* 103(2): 169-77
14. Maccarrone, M., Bari, M., Battista, N., Finazzi-Agro, A. (2001). The catalytic efficiency of soybean lipoxygenase-1 is enhanced at low gravity. *Biophysical Chemistry* 90: 97-101

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