Drew University

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Effect of Ca^{2+} ions on the hydrolysis of phenyl esters and anilides, *p*-nitrophenyl acetate and *p*-nitroacetanilide

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by

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Abstract

Enzymes are used for a number of industrial and medicinal reasons. To study enzymes, small model systems are frequently used to simplify the catalytic systems that enzymes use. There has been studies done metal ions and phosphodiesters, Ca^{2+} ions on phosphodiesters, metal ions and esters. But, one particular field of model systems not studied much is the effect of Ca^{2+} ions on esters. This would give us a broader understanding in which Ca^{2+} ions are able to interact with carbonyl oxygens in models, even if they do not exhibit an negative charge. In order to test the importance of the negative charge on the non-bridging oxygens in phosphodiesters for metal ion catalysis, the effect on hydrolysis rates with Ca^{2+} was examined for *p*-nitrophenyl acetate (*p*NPA). This was tested by running kinetic assays by measuring the appearance of the product, *p*-nitrophenolate, using UV-Vis spectroscopy and also varying both the [$^{-}$OH] and [Ca^{2+}]. Through these assays, it was found that Ca^{2+} did indeed catalyze the hydrolysis from *p*NPA to *p*-nitrophenolate, and only one Ca^{2+} was involved in the catalysis. The rate constant with respect to Ca^{2+} was found to be 1.51 M/M^2s^{-1}, and that using a [Ca^{2+}]=330 mM increases the rate of hydrolysis by approximately 77% compared to no Ca^{2+} ions, 1131 m/Ms^{-1} and 638 m/Ms^{-1} respectively. These data indicate that although there is an increase in hydrolysis rates for *p*NPA when Ca^{2+} ions are present, it is very small compared to phosphodiesters, whose catalysis can be over 800 fold. This could be due to several different factors, including the negative charge on the phosphodiesters having a larger interaction with Ca^{2+} than a carbonyl oxygen Ca^{2+}.

In order to test how Ca^{2+} ions catalyze the hydrolysis of *p*NPA specifically, the molecule *p*-nitroacetanilide (*p*NAA) was examined. Where *p*NPA's hydrolysis occurs via a concerted fashion, *p*NAA's occurs via a stepwise reaction. This would allow us to see if Ca^{2+} interacts with the carbonyl oxygen or only the nucleophile. *p*NAA's tetrahedral intermediate contains a

negatively charged non-bridging oxygen that could theoretically interact with Ca^{2+}, thus leading to an increase in catalysis. *p*NAA was found to only have a 2.64 fold catalysis, which compared to *p*NPA's 1.77, is not that significant either. This leads to the belief that Ca^{2+} catalyzes the hydrolysis of both *p*NPA and *p*NAA by coordinating with the nucleophile, hydroxide, rather than interacting with the carbonyl oxygen.

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1.0 Introduction

1.1 Enzyme Function and Impact

Enzymes are used for industry and medicine, which is why they have been studied extensively since the early 1800s[1]. Enzymes can be used to speed up chemical reactions that would normally be expensive and timely. It is not uncommon for an enzyme to increase the rate of the reaction by as much as 10^{10} fold. Studying enzymes is the key to understanding how to catalyze reactions at these high rates[2]. Also, a popular field of research right now is site-directed mutagenesis to maximize the efficiency and uses of enzymes in industry and medicine[1]. Studying about enzymes is also important to understand how they work. Some drugs use enzymes as targets, so learning how they work will make it easier to develop these kind of drugs.

Emil Fisher was one of the first scientists in the field to explain how enzymes work to catalyze reactions[2]. His way of explaining this was with the lock and key hypothesis, where the substrate is the key and the enzyme is the lock, suggesting that there is only a few substrates that can fit inside the enzyme's binding pocket, like a key and a lock. If the substrate fits inside, the reaction will be catalyzed. The "key and lock" explains generally why enzymes are specific. It does not explain why enzymes catalyze reactions so efficiently. The reason why enzymes increase reaction rate can be explained the fundamental principles of physical chemistry[1]: absolute reaction rate theory and the thermodynamic cycle. Over billions of years of evolution, enzymes have developed to be perfect catalysts for the reactions they are catalyzing. The way that an enzyme will catalyze the reaction is through transition-state stabilization. This occurs when an enzyme binds to the transition states of the reaction and it increases the reaction rate proportional to the increase in concentration. It is important that the transition state binds much more strongly to the enzyme than

the ground state does so that the energy required to reach the transition state decreases. The change in energies between these two is equal to the enzymatic rate acceleration (Figure 1).

Enzymes and substrates can exist in solution together in a few different ways depending on whether the enzyme can interact with the substrate or not. The relationship between the substrate and enzyme will lead to two different pathways through which a reaction can occur (Figure 1A) one being the non-catalyzed pathway and the other being the catalyzed pathway. Even when the enzyme and substrate are compatible, if there is not enough enzyme present compared to the substrate, some of the substrate will be uncatalyzed and still react but more slowly. When the enzyme and substrate are in solution together, the first step is the enzyme and substrate forming a complex with one another. Then the substrate will turn into its transition state because that is when the enzyme binds most tightly to the substrate. After the enzyme and the substrate's transition state are formed in a complex, the substrate will convert to the final product and be released from the enzyme, leaving the enzyme and product separated in solution. The bottom pathway will occur faster than the top pathway because the [$ES^{‡}$] complex is more stable than E + $S^{‡}$, due to the enzyme binding tighter to the transition state than the ground state.

The energetics of the scheme can be explained by an energy diagram of uncatalyzed and catalyzed reactions (Figure 1B)[3]. The ΔG for both reactions are the same because both contain the same reactants and products. However, the activation energies, denoted by $\Delta G^{‡}$ are different for the reactions. The uncatalyzed reaction's $\Delta G^{‡}$ is far greater than the $\Delta G^{‡}$ of the catalyzed reaction. Also, the catalyzed reaction is split up into several different steps with multiple intermediates and the uncatalyzed reaction contains only one intermediate. The increased number of intermediates in the catalyzed reaction is the key to why there is a reduced activation energy for the reaction.

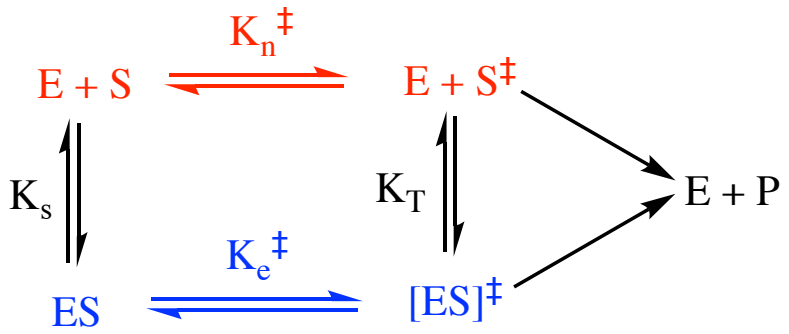


Figure 1A Different interactions that an enzyme and substrate can have in solution together. Modified from Kraut[1]. E = Enzyme, S = Substrate, P = Product, and ‡ indicates Transition state. The pathway on the top indicates the non-catalyzed reaction. The pathway on the bottom indicates the catalyzed reaction. The (+) indicates that the enzyme and substrate/product are not bound together. The equilibrium labeled K_T is only theoretical to illustrate relative affinity, $S^{\ddagger}$ does not exist long enough to find E. Figure 1B helps illustrate the energies of each state. In this figure $K_T >> K_S$ and $K_e^{\ddagger} >> K_n^{\ddagger}$.

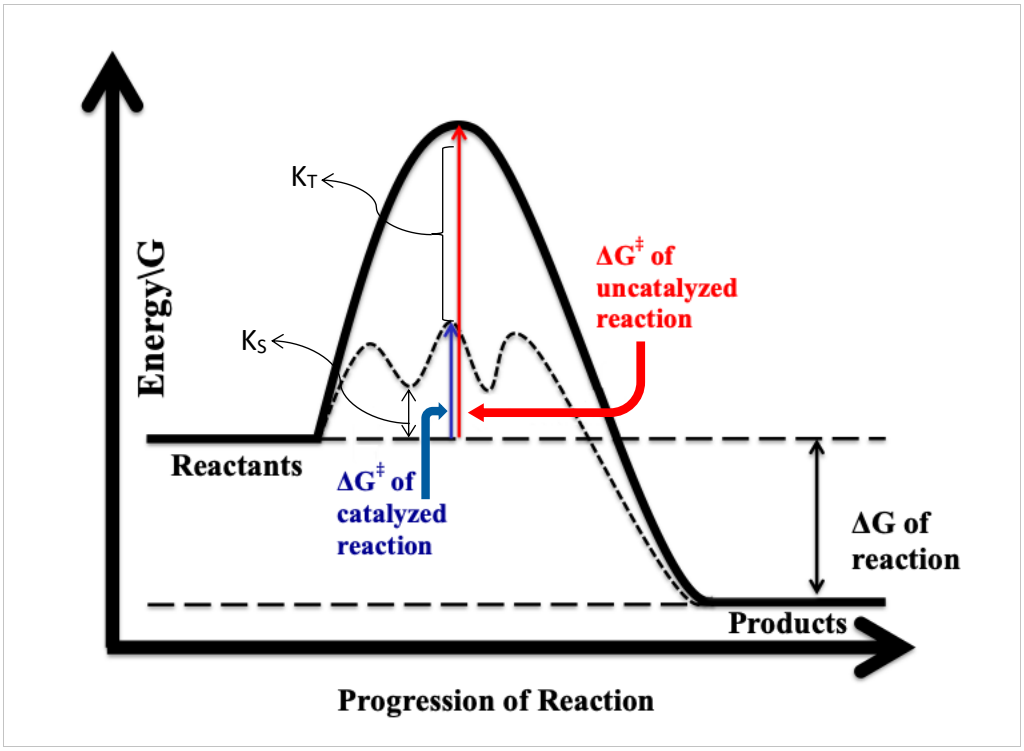


Figure 1B Energy diagram of a non-catalyzed reaction versus a catalyzed one[3]. The red arrow indicates the activation energy needed for an uncatalyzed reaction. The blue arrow indicates the activation energy needed for a catalyzed reaction to occur.

Studies on enzymes have shown that they undergo conformational changes in order to speed up reactions[2]. There are a few reasons why this may occur, one being that there are multiple intermediates in the reactions that they catalyze. With each intermediate, it is believed that the enzyme will change shape to accommodate the next intermediate, and bind even more strongly, or make the following step in the reaction more likely to occur[2]. Another reason why a conformational change may be favorable for enzymes is because the enzyme breaks a reaction into several steps. All of these steps have lower free energies of activation compared to the single step reaction. In order for this to occur for different steps of the reaction, the enzyme must have conformational adaptability, changing its structure for each step of the reaction[2]. Due to the conformational adaptability for the several step chemical reaction, enzymes need to be fairly large macromolecules (at least 10,000 Da).

There are many classes of enzymes, but when it comes to the energy changes, they follow the same trends. One class of enzymes are metalloenzymes, where there is a metal ion that is either being used for a catalytic, cocatalytic, and/or structural, role in the enzyme[4]. The most popular metal ion used in metalloenzymes is zinc because it is able to act as a Lewis acid.

Zinc ions are often present in metalloenzymes and are responsible for the catalytic properties of the enzyme[4]. In order for zinc metalloenzymes to catalyze the reaction, there needs to be a zinc-bound water present in the catalytic site. This zinc-bound water has one of three functions: 1) it can be ionized to form a zinc-bound hydroxide ion, 2) it is polarized by a general base to form a better nucleophile, and 3) water can be displaced by the substrate (Figure 2). When the zinc ion is used as an electrophile, it can catalyze the reaction in several ways, either by activating the water molecule for nucleophilic attack, stabilizing the carbonyl of the scissile bond through polarization, stabilization of the negatively charged transition state, or any combination of the three. It is also

important to note that although zinc ions are the most popular metal found in enzymes, other metal ions, including Mg^{2+} and Ca^{2+}, act in the same way as zinc ions do.

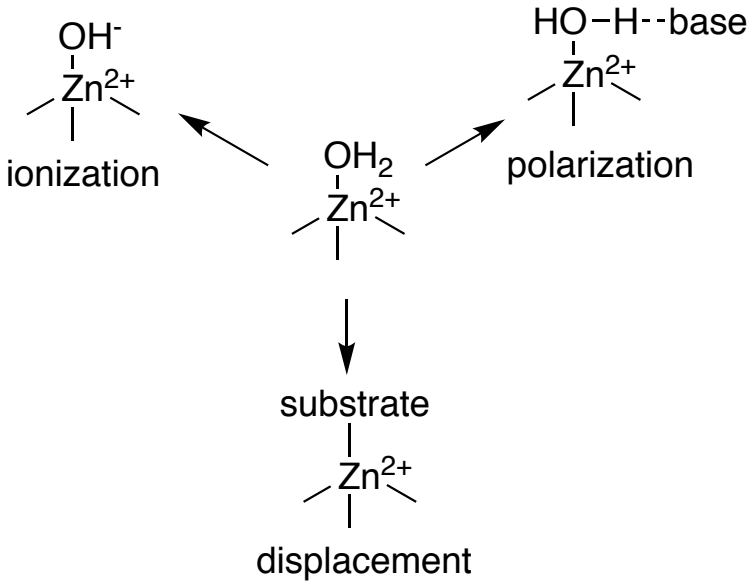


Figure 2 Different functions that zinc-bound water can have. The zinc-bound water can be ionized, polarized, or displaced by a substrate.

1.2 Phosphodiesterases

DNA and RNA are molecules that are responsible for the makeup of the body and the reason why characteristics are inherited from generation to generation[5]. DNA and RNA are stable due to the phosphodiester bonds. Phosphodiester bonds are part of the backbone in both RNA and DNA and hold the sugars and nucleotide bases in place. These bonds normally have some of the lowest rates of cleavage in biology, with DNA having a half-life at around 30 million-years[5, 6]. The stability is why these bonds are found throughout many biological molecules. However, some enzymes, particularly phosphodiesters and nucleases are able to break these stable phosphodiester bonds easily, in order to repair DNA, regulate signaling, and process RNA[7]. Additionally, other phosphoryl transfers are extremely important for biology[5]. Basic metabolic pathways as well as

cellular signals require phosphoryl transfers. For these reasons, studying phosphodiesters and phosphoryl transfers in general is extremely beneficial to society.

Many phosphodiesterases utilize metal ion cofactors. The metal cofactors are why phosphodiesterases are so active; all of the negative charge built up on the non-bridging oxygens on phosphodiester bonds are stabilized by the positive charge on metals. It is important to understand the role these metal ions play because there are a lot of different enzymes that utilize divalent metal ions, but they all have a varying number of metal ions[8]. Previous work has showed that divalent metal ions play a dual role in the hydrolysis of phosphodiester bonds. One is stabilizing the nucleophile, OH^-, and the other is stabilizing the negative charge that is transferred from the OH^- to the phosphate. Studies on DNA polymerase I showed that two of the divalent metal ions, Mg^{2+}, are being used for catalyzing the hydrolysis of phosphodiesters (Figure 3). What was found that the Mg^{2+} interacts with the nucleophile, OH^-, and the substrate, phosphate, the same way as previously thought. However, the difference is that one Mg^{2+} is being used for stabilizing the OH^- and a different Mg^{2+} was being used to stabilize the negative charge that was transferred from the OH^- to the phosphate (Figure 4).

A few mechanisms for DNA cleavage with an external nucleophile were suggested while studying the endonuclease *Eco*RV (Figure 5)[9]. The first proposed mechanism (Figure 5A) uses a metal ion to stabilize the negative charge formed on the water molecule after it donates a proton to the leaving group, from the nucleophilic attack of a hydroxide. The second proposed mechanism (Figure 5B) uses two metal ions in the mechanism, the first stabilizes a hydroxide ion to be used for a nucleophilic attack on the phosphate group and the second metal ion stabilizes the negative charge on the phosphate group. The metal ion does decrease the nucleophilicity of the bound hydroxide ion, however the metal bound hydroxide is still a better nucleophile than a water

molecule. Both of these mechanisms would be plausible for how *Eco*RV cleaves phosphodiester bonds. Later research with *Eco*RV showed that there are both associative and dissociative pathways found for the enzyme to hydrolyze phosphodiester bonds[10]. For both of these types of pathways however, Mg^{2+} was found to be playing the same role. Mg^{2+} was suggested to be interacting with both the phosphodiester's non-bridging oxygen, as well as a water molecule. The only difference between the associative and dissociative pathways is that one was activating the water molecule to be used as a general base, and the other pathway was using the water molecule as a general acid.

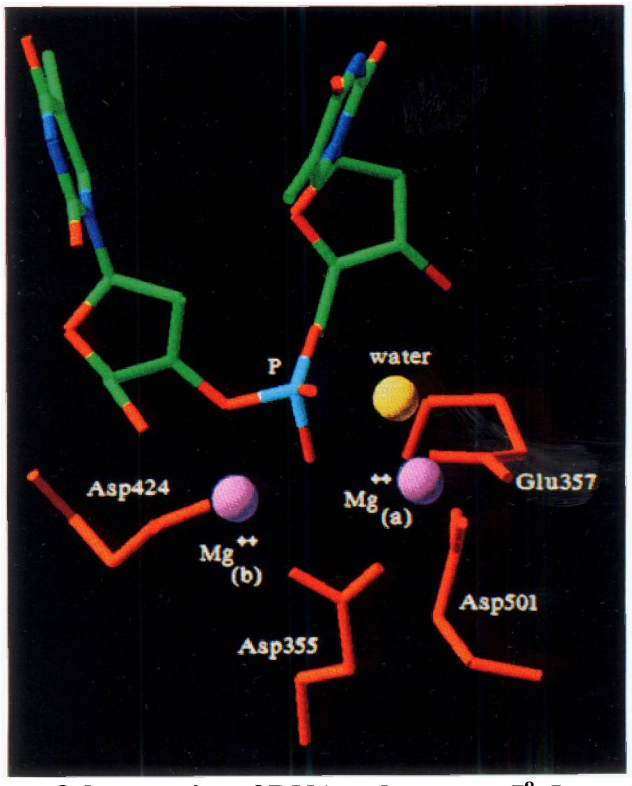


Figure 3 X-ray structure of the exo site of DNA polymerase I[8]. It can be seen that the exo site utilizes a water molecule as well as two Mg^{2+} ions in its active site, which aids in the hydrolysis of phosphodiester bonds.

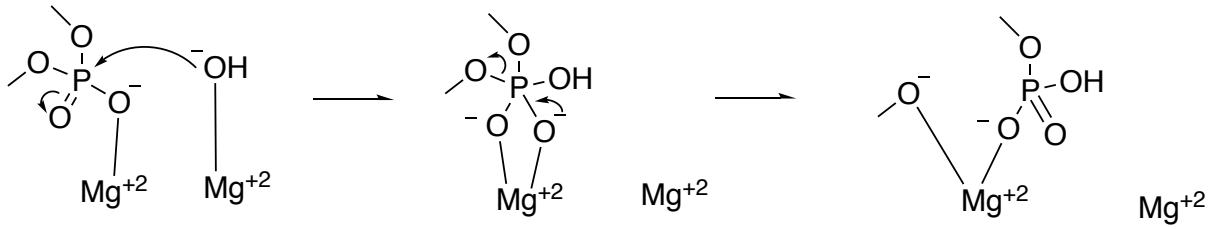


Figure 4 Two Mg^{+2} ion mechanism of the hydrolysis of phosphodiesters in DNA polymerase I[8]. One Mg^{2+} ion is used to stabilize the negative charges that were formed on the phosphate after the attack of a hydroxide ion. The other Mg^{2+} ion was used to stabilize the nucleophilic hydroxide ion.

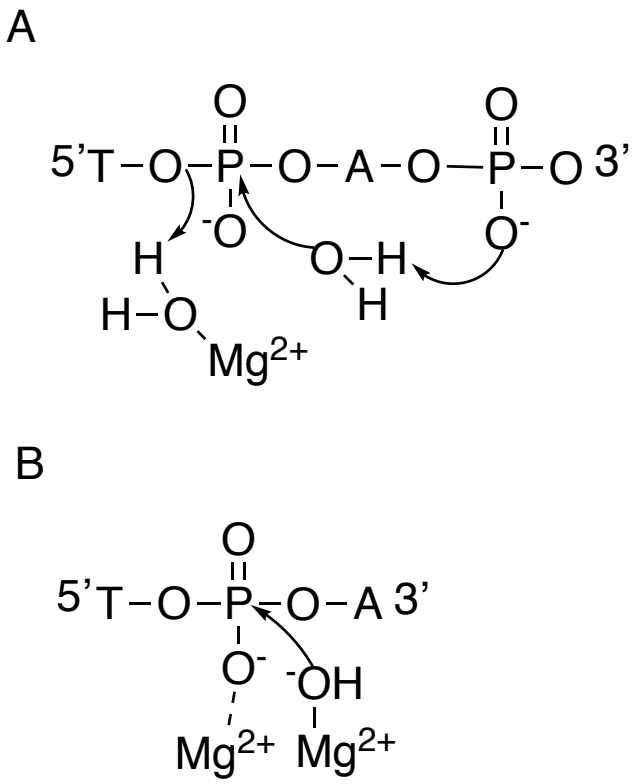


Figure 5 Metal ion catalyzed hydrolysis of DNA with an external nucleophile in *Eco*RV. Modified from Groll et.al[9]. Two plausible mechanisms in which the endonuclease *Eco*RV can cleave phosphodiester bonds between a thymine and an adenine. (A) A Mg^{2+} ion is used to activate a water molecule which is then used as an acid to stabilize the phosphate group that will be cleaved through general acid catalysis. (B) Two Mg^{2+} ions are being used together to help cleave the phosphodiester, one is stabilizing a hydroxide ion to be used for nucleophilic attack and the other is stabilizing the negative charge on the phosphate group.

In both the RNA hydrolysis (Figure 6) and DNA hydrolysis (Figure 5) a metal ion activates a water molecule to aid in the hydrolysis of phosphodiesters[9]. The mechanisms differ however because the RNA model uses its internal 2' hydroxyl group to attack the phosphate group (Figure 7). *Eco*RV utilizes an external nucleophile, in the form of water to attack the phosphate group. These differ because intramolecular reactions are more likely to occur, therefore be faster, than intermolecular reactions. We would like to focus on the external nucleophile because DNA does not have a 2' hydroxyl which is why the hydrolysis half-life is significantly longer than RNA's (30 million years versus 10 years) (Figure 7)[6]. In order to see the dramatic increase in hydrolysis rates using metal ions, external nucleophiles should be used because the reactions occur very slowly without the help of a catalyst.

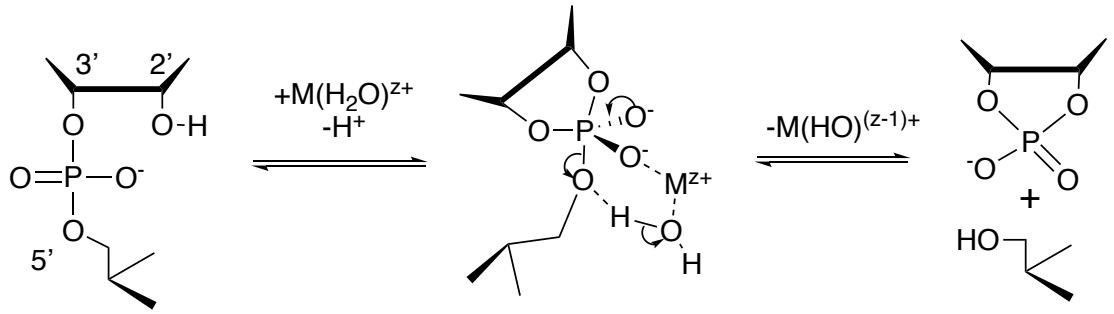


Figure 6 Metal ion catalyzed hydrolysis of a phosphodiester in RNA with an internal Nucleophile. Modified from Lönnberg et.al[11]. The cleavage of the RNA backbone is successful due to metal ion catalysis. The metal ion chelates to the non-bridging oxygen and activates a water molecule, to be used as an acid. The water molecule protonates the bridging oxygen, thus cleaving the phosphodiester.

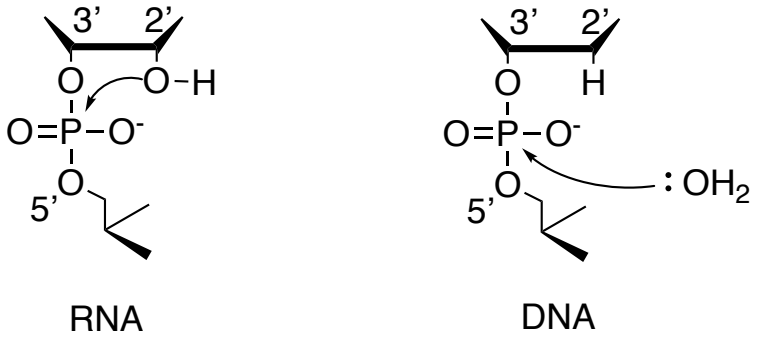


Figure 7 The hydrolysis mechanisms of RNA and DNA cleavage. The phosphodiester backbone in RNA is cleaved intramolecularly using the 2' hydroxide. DNA does not have a 2' hydroxide so therefore must be cleaved intermolecularly with an external nucleophile.

1.3 Ester Hydrolysis with Enzymes

Another important class of metalloenzymes consist of esterases. The biggest difference between phosphodiester and ester structure is that phosphodiesters exhibit a negatively charged non-bridging oxygen. Esters have a non-bridging oxygen as well, but this oxygen is found in the carbonyl, therefore it is not charged (Figure 8). Some of the first esterase structures that were found crystallographically were the zinc proteases, carboxypeptidase A_α and thermolysin[1], enzymes that catalyze hydrolysis of amide and ester bonds. Carboxypeptidase is a digestive enzyme found in the pancreas of vertebrates, and thermolysin is found in the bacterium *Bacillus thermoproteolyticus*. Thermolysin is an endopeptidase that hydrolyzes the peptide bond specifically before large hydrophobic residues. Carboxypeptidase is an exopeptidase that is specific to large hydrophobic residues at the carboxyl terminus. These proteases look different by both tertiary structure and by amino acid sequence. However, both of these enzymes utilize a Zn^{2+} ion at their reactive centers, along with two His side chains, one Glu side chain, and a water molecule. These two enzymes are proposed to have the same mechanism of hydrolyzing peptide bonds. First, the carbonyl oxygen of the peptide coordinates with the zinc ion, which in turn brings the zinc-water complex toward the carboxylate group on the Glu residue. This is similar to how phosphodiesterase metal ions interact with the negative charge on the non-bridging oxygen atoms. Then, with help of both the carboxylate group and the zinc, the water molecule is activated and attacks the carbonyl carbon on the peptide, forming a tetrahedral species. The water then donates one of its protons to the leaving group nitrogen, cleaving the peptide bond (Figure 9).

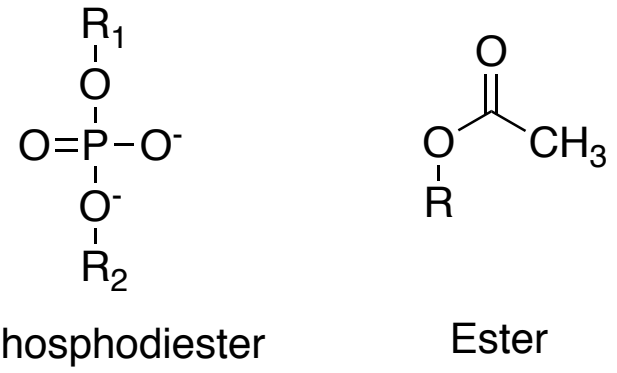


Figure 8 Structure of phosphodiesters compared to esters. Phosphodiester bonds consist of two bridging oxygens and two non-bridging oxygens, where one of the non-bridging oxygens houses a negative charge. Esters consist of one bridging oxygen and one non-bridging oxygen, and this non-bridging oxygen does not have a negative charge.

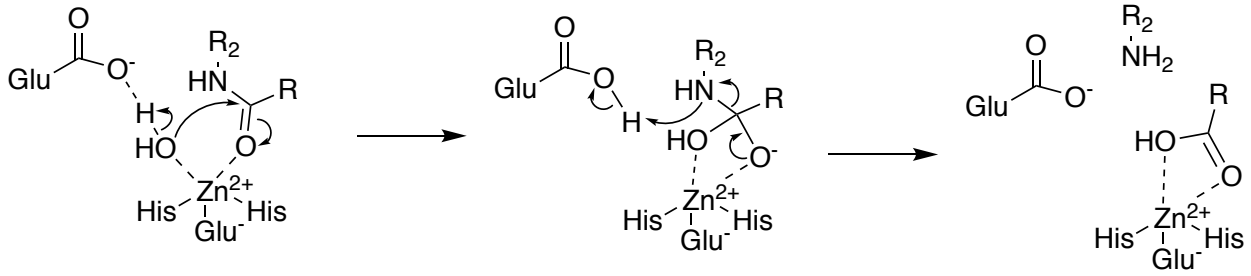


Figure 9 Mechanism of peptide hydrolysis of carboxypeptidase and thermolysin[1]. Both of these enzymes hydrolyze peptides similarly. Both utilize a zinc ion to interact with the carbonyl and a water molecule. The water molecule attacks the carbonyl carbon and a Glu is used as a proton acceptor then donor to help stabilize the charges on the molecules.

Other than peptidases, there are other zinc metalloenzymes such as hydrolases and there has been research done to better understand the mechanism of zinc-dependent deacetylation[12]. The mechanism of the enzyme UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase (LpxC) was looked more in depth to study zinc hydrolases[12]. There were two proposed methods of the enzyme catalyzing the deacetylation of substrates (Figure 10). The first proposed mechanism of catalysis is similar to carboxypeptidase A (Figure 10A). A Glu residue acts as a general base and activates a nucleophilic zinc-water complex, for attack on the carbonyl carbon on the substrate. It is also believed that the zinc ion has a second role, to stabilize the tetrahedral intermediate by polarizing the ground state by interacting with the carbonyl oxygen. The second proposed

mechanism involves the zinc bound water molecule nucleophile again, except, now the zinc is interacting with the carbonyl oxygen to polarize the ground state (Figure 10B). Again, the Glu residue acts as a general base to activate the zinc-water complex for nucleophilic attack of the carbonyl carbon. The zinc ion is still used to stabilize the tetrahedral intermediate and the transition state. This research suggests that there are several different ways metals play a role in catalysis for metalloenzymes. The fact that it is not really known for sure how a lot of enzymes work mechanistically reassures why it is important to continue studying the mechanisms of enzymes, and in this case, metalloenzymes.

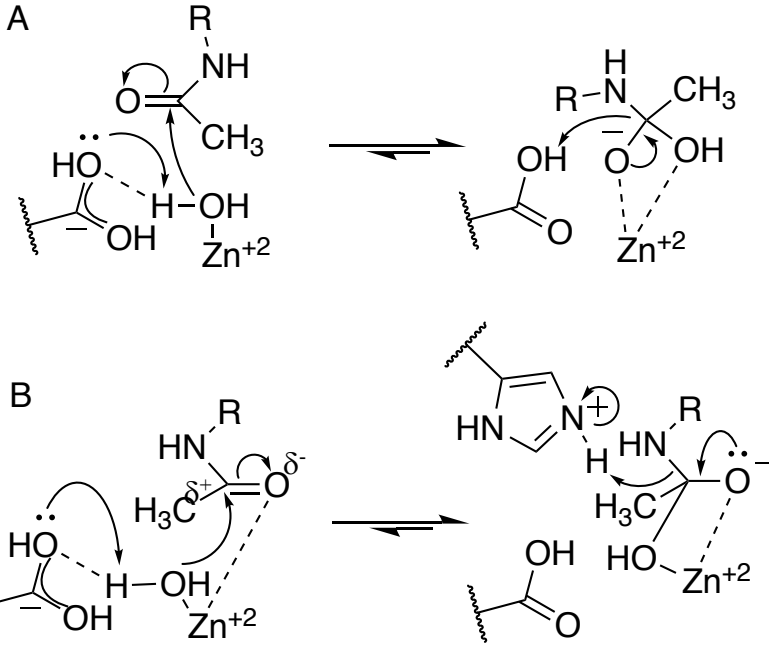


Figure 10 Two proposed mechanisms of zinc ion hydrolases of esters/peptides[12]. Two possible mechanisms in which zinc ion hydrolases can hydrolyze esters. Both of the mechanisms utilizes a zinc bound water molecule that gets deprotonated via a Glu residue and attacks the carbonyl. (A) The zinc ion interacts with only the water molecule until after the nucleophilic attack, and then the zinc helps stabilize the negative charge formed. (B) the zinc ion interacts with both the carbonyl oxygen and water molecule to coordinate the nucleophilic water to attack the carbonyl.

Enzymes utilize metal ions other than Zn^{2+} as well, one example was found while examining the mechanism of action of guinea pig liver transglutaminase[13]. They found that this enzyme is able to catalyze the hydrolysis of *p*-nitrophenyl acetate (*p*NPA) but only when a divalent

metal ion is present, such as Ca^{2+} ions. Previous work with this enzyme showed that calcium ions were needed to catalyze a hydrolysis for amides, so they decided to redo the experiment, except with *p*NPA. They tested the velocity of *p*NPA hydrolysis of transglutaminase at different calcium concentrations. They found that the metal ion combines with the enzyme forming a metal enzyme complex. The final conclusion was that *p*NPA was hydrolyzed through a single cation-controlled mechanism. As they increased the calcium concentration, the velocity of the enzyme also increased, as they showed through a Lineweaver-Burke plot. It was found that the enzyme gets activated by a metal ion binding at two different spots of the enzyme. One site is responsible for the binding of the substrate. The metal ion at the other site is essential for catalysis. This research is important for me because I am studying the effect of Ca^{2+} ions on the hydrolysis of *p*NPA. Therefore this experiment and my experiment are very similar, except their's is using an enzyme and mine is in a basic aqueous solution.

Another enzyme that was found with esterase ability is serum paraoxanase (PON1), and is found in mammals and other vertebrates[14]. The interesting thing about this enzyme is that its catalytic ability comes from two Ca^{2+} ions within its active site. Not only that, but the way in which the enzyme's Ca^{2+} ion catalyzes both esters and phosphoesters is similar to how it is believed that Ca^{2+} can catalyze *p*NPA in our model system. This enzyme is usually used to hydrolyze (therefore inactivate) different organophosphates, which include nerve agents like sarin and soman. Because of the fact that these organophosphates are being used for chemical warfare and terrorism, there has been more research on this particular enzyme recently. One Ca^{2+} ion is used for the catalytic ability and the other Ca^{2+} ion is used entirely for the structure of the enzyme. They were able to determine the enzymatic mechanism for the ester, 2-naphthyl acetate, and the phosphotriester, paraoxon. What they found was that the catalytic Ca^{2+} interacts with the carbonyl or phosphoryl

oxygen, holding the molecule in place, then a water molecule that has been deprotonated by a histidine attacks the carbonyl carbon, thus hydrolyzing the molecule (Figure 11). Studying this enzyme is of importance to us because this enzyme utilizes Ca^{2+} ions and shows that they are needed for this catalysis, not only for catalyzing the hydrolysis of phopshoesters but also esters. It would be important to learn if there is a difference in levels of catalysis between these two classes of molecules. This would explain how Ca^{2+} is coordinating with oxygens in different molecules. Therefore, using *p*NPA, an ester, similar to 2-napthyl acetate, would be important to study in a Ca^{2+} model system, and compared to phosphoesters.

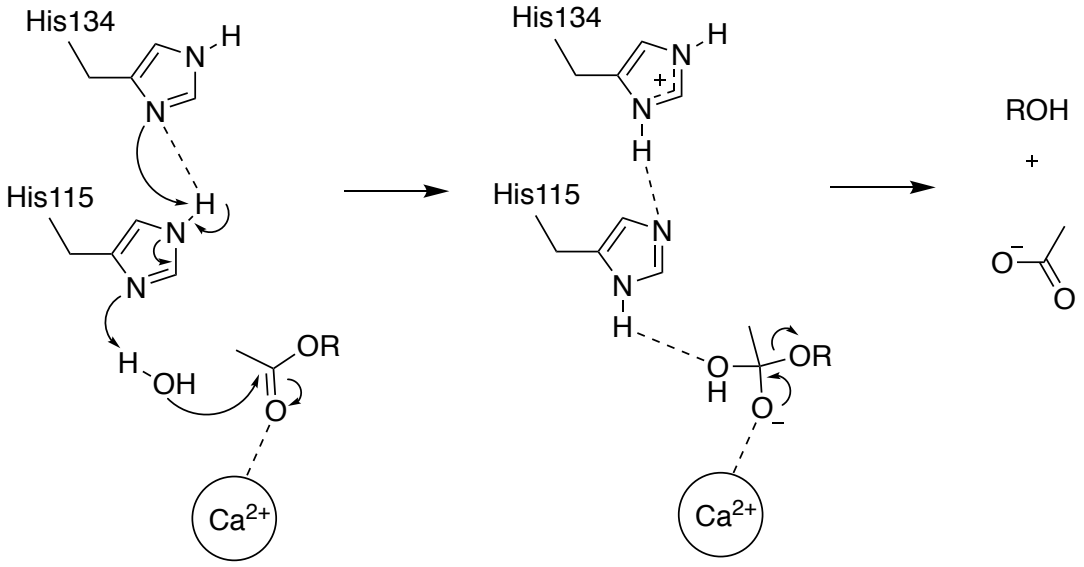


Figure 11 Proposed mechanism of ester hydrolysis for the esterase PON1[14]. Histidine 115 is being used to deprotonate a water molecule to form a hydroxide which is then used as a nucleophile to attack an ester. Histidine 134 is being used to make histidine 115 a better base. A Ca^{2+} ion is used in the active site to interact with the carbonyl oxygen to keep the ester in place to help hydrolysis.

1.4 Model Systems

As stated previously, a metal ion can play several roles in metalloenzymes, either catalytically, cocatalytically, and/or structurally[4]. When the metal ion is used in the catalytic site, the ion is responsible for assisting in the bond-breaking and -forming during the reaction. A cocatalytic ion will enhance the catalytic properties of another metal ion in the catalytic site.

Structural metal ions participate in stabilizing the tertiary structure of the enzyme. No matter what role the metal ion plays for the metalloenzyme, if the metal ion is removed, the catalytic properties of the enzyme can be lost.

The biggest problem with studying the role of metals in enzymes is that it is very complicated to figure out if the metal plays a catalytic, a structural role, or both. The reason being that if the metal is taken out, the enzyme will lose its activity whether it is playing a structural or a catalytic role. It is hard to focus on one particular aspect of the enzyme when working directly with it. For that reason, model systems are often used instead. This way, a specific part of the enzyme can be studied and altered to see the results. For example, to test the importance of a metal ion center in the enzyme, the metal ion can be in solution with the reactants, and you can test the rate of reaction. If you want to see if the metal ion is catalyzing the reaction, the metal ion concentration can be increased. There are also other aspects that can be studied easier in a model system than with enzymes. These aspects can be changing the identity of the metal or changing the reactivity of the substrate or even the entire substrate. When changing the metal ion present in solution, the reaction can be rerun except in presence of the other metal. With enzymes, the metal binding sites may not work with different metals. Also, the same experiments could be ran with either the same substrate but with different reactivity, or a totally different substrate. With model systems, all of this is easy to study because they are not as complicated to manipulate as enzymes are.

Model systems have been used since the early 1800s as a better way to study enzyme action[15]. One of the main reasons to use model systems as opposed to enzymes is that there is no need to worry about the structure of the enzyme. With enzymes, changing a few amino acids may completely change the shape of it, which could possibly make the enzyme cease to work. But, it

will still be unknown if the function was loss due to the overall structure of the enzyme, or just the active site affected. With metal ion catalysis, model systems are used to determine the role/s the metal/s play/s in the enzyme. Model systems can also be used to give a baseline for how metal ions promote a reaction and how much energy can be contributed towards transition state stabilization. If you take the enzyme out of the equation and use the metal ions by themselves for the reaction, you can determine the role the metal ion plays in the enzyme[15]. However, if you take the metal ion out of the enzyme, the enzyme could stop working, but you will not know if it was because the metal played a catalytic or structural role. Upon removal of the metal ion, there is no catalysis, than the metal ion could have played a structural role in the enzyme. However, if the metal ion in solution catalyzed the reaction, then it is believed that the metal ion plays a catalytic role in the enzyme. If there is a huge catalytic defect upon removal of the metal, combined with the modest catalysis with the metal alone, may suggest that the metal plays a dual role structurally and catalytically.

As in metalloenzymes, metal ions acting as Lewis acid catalysts in aqueous solution can have several different ways that the metal ion can catalyze the reaction. Thus, studying how these catalysts act in aqueous solution can provide information to their role in an enzyme active site. There are also different types of catalysts that will increase the reaction rate: hydroxide ion catalysis and general acid base catalysis[16]. Hydroxide ions can catalyze the reaction in one of two ways; one way is by converting the substrate to its conjugate base, then the substrate reacts **(**Figure 12A) and the other way is that the substrate will combine with the hydroxide ion and the transition state will include both the substrate and hydroxide ion (Figure 12B). General acid base catalysis occurs when the rate of reaction is proportional to the amount of the acid or base in solution[16]. What this means that if a base is responsible for catalysis, as you increase the concentration of the

base in solution, the rate of reaction will also increase at the same rate, the same goes with if acids are used. Both of these modes could occur in the active site of an enzyme.

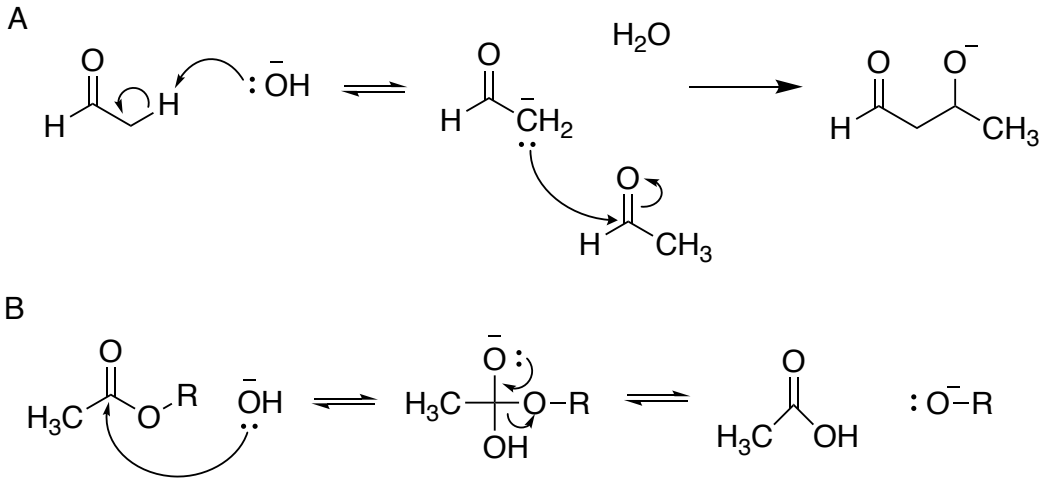


Figure 12 Different ways that hydroxide ions catalyze reactions. Two mechanisms in which hydroxide ions catalyze reactions. (A) A hydroxide ion is used to convert the substrate into its conjugate base and the conjugate base reacts. This particular reaction is an aldol reaction[17]. (B) A hydroxide ion combines with the substrate and the transition state contains the substrate and hydroxide ion.

When metal ions play a role as Lewis acids in metalloenzymes, there are several different ways in which the metal ion can catalyze the reaction[18]. It is important to note that the most common and important metal ion for metalloenzymes is zinc (II) and is seen to catalyze almost all organic reactions. It has also been seen that metalloenzymes are capable of activating electrophiles, such as carbonyl, phosphoryl, and nitrile groups, as well as several leaving groups. 6 different methods in which a metal ion can catalyze a reaction in metalloenzymes was found (Figure 13). (1) The metal ion in the active site binds to a water molecule or a hydroxide, and the metal-bound water/hydroxide acts as a nucleophile and attacks the substrate, hydrolyzing it (Figure 13). (2) The metal ion binds to a water molecule, making the water molecule act as a general acid, which can then protonate the leaving group, making it more stable and likely to leave (Figure 13). (3) When a metal ion binds to a hydroxide ion, the hydroxide ion acts as a general base and can lower the

energy needed for the formation and breakdown steps of the intermediate (Figure 13). (4) The use of two metal ions in the active site, which may catalyze reactions due to the geometry of the transition state, but the exact mechanisms were not known at the time, and rarely found in metalloenzymes (Figure 13). (5) A metal ion bound to a specific part of the substrate could prevent reverse reactions from occurring, by blocking it, and therefore catalyzing the main reaction (Figure 13). (6) The metal ion is capable of coordinating with other organic catalytic functional groups that can further increase the speed of the reaction (Figure 13). The most relevant of these mechanisms to study metal ion interactions with the carbonyl will be the metal bound water molecule acting as a nucleophile.

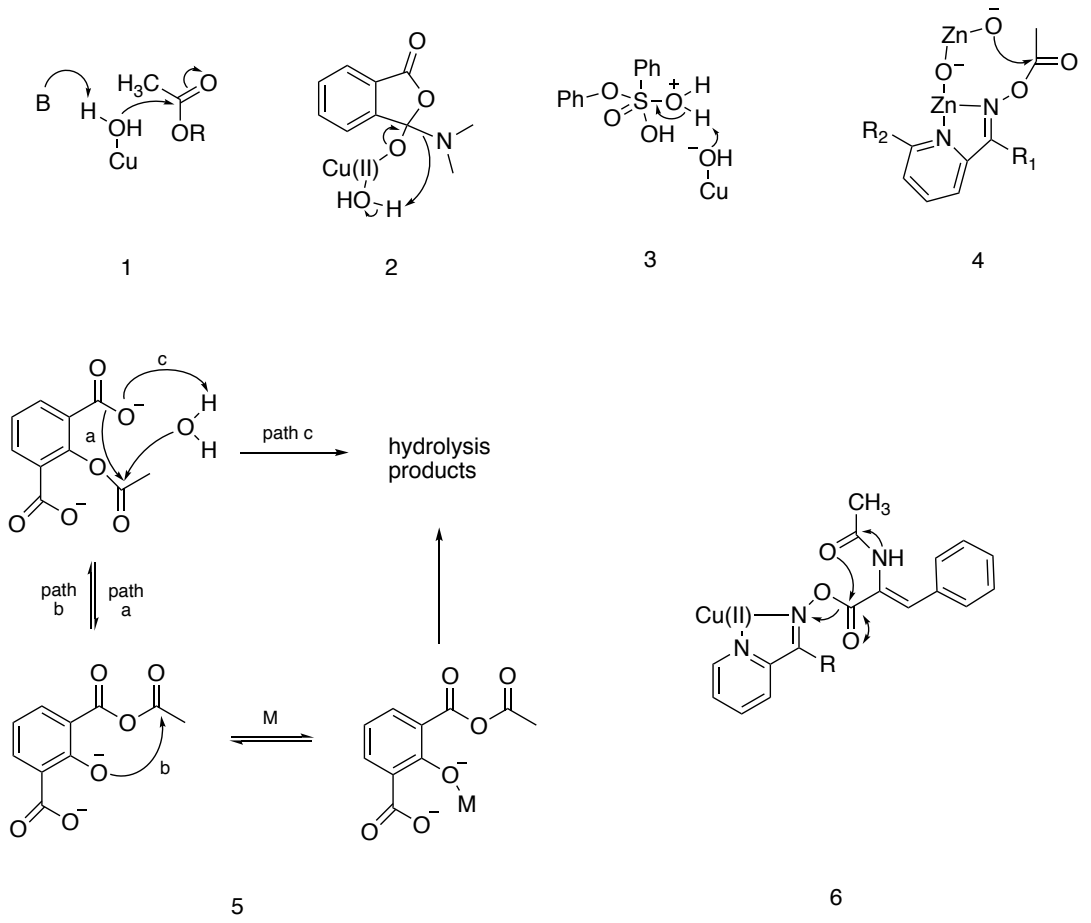


Figure 13 Six different ways in which metal ions can catalyze a reaction in a metalloenzyme[18]. (1) Metal-bound water molecule as a nucleophile. (2) Metal-bound water molecule as a general acid. (3) Metal-bound hydroxide ion as a general base. (4) Two metal ions increase electrophile by acting as Lewis acids. (5) Metal ion blocks a reverse pathway. Path a is the first step in the reaction and path b is the reverse of it. After path b, path c occurs to hydrolyze the product. A metal ion can bind to the substrate to prevent path b from occurring, forcing the final hydrolysis reaction to occur. (6) A metal ion can cooperate with organic functional groups. The Cu(II) ion is activating the leaving oximate ion.

1.5 Phosphodiesterase Model Systems

DNA and RNA molecules are highly stable, with the half-life for the hydrolysis of DNA's phosphodiester bond being around 30 million years, and the half-life for RNA's being around 10 years[6]. However, with the help of DNases and RNases, the hydrolysis of the phosphodiester bonds are increased by 10^{17} fold and 3 x 10^{11} fold, respectively. Because these enzymes catalyze the

reaction so well, phosphodiester cleavage is very popular to investigate, using small molecular models. This allows for easier studies on the transition states, proton transfers, metal ion binding, and other characteristics of an enzyme. These studies are almost impossible to do with actual enzymes because the smallest change in the enzyme's protein structure could severely alter the structure or function of the enzyme. Also, many of the enzymes that catalyze phosphodiester cleavage are ribozymes (catalytic RNA). These are even harder to compare structure to catalytic effects because metal ions play a huge role in shielding the negatively charged backbone from itself.

For RNA models, the mechanism for one model of metal ion catalysis is that the metal cation interacts with water and the negatively charged oxygen on the phosphate group, which allows the water to undergo a general acid catalysis, thus breaking the phosphodiester backbone into two (Figure 6)[11]. Other research proposed several different models for how metal ions can bind to phosphodiesters[19]. It was proposed that the metal ion can interact with either one or two of the negatively charged oxygens during the transition state of phosphodiester hydrolysis. It was also proposed that the metal ion can also interact with both a negatively charged non-bridging oxygen as well as the attacking hydroxide.

Previous studies on RNA hydrolysis was done using the model molecule, 5-Uridine-Guanosine-3'[20]. This model system also used several different isomers of this molecule as well, the R_P and S_P phosphorothioate analogs, where one of the non-bridging oxygens was replaced by a sulfur. What was found using these model molecules is that these RNA analogs does indeed get catalyzed by Ca^{2+} ions. One Ca^{2+} ion is used to catalyze the reaction by interacting with the negatively charged non-bridging oxygen. This led to an increase in catalysis of 326-fold. The R_P analog was found to have a 129-fold increase and the S_P analog had a 68 fold increased. The

analogs had a smaller fold increase because the Ca^{2+} does not have as strong of an interaction with sulfur compared to oxygen.

Another field of research for phosphodiester hydrolysis is using compounds with multiple ligands (aromatic ring nitrogen atoms and hydroxyl groups) that are able to form a complex with multiple metal ions in order to test for metal ion catalysis[21]. They used several different ions such as La^{3+} and Zn^{2+} to test the effects of metal ions on the hydrolysis of phosphodiesters as well as different types of ligands. They tested the effects of La^{3+} ions on the hydrolysis of the phosphodiester, (8-hydroxy-2-quinolyl)methyl (8-hydroxyquinolyl)-methylphosphonate because two metal ions are able to ligate to this molecule and will cancel the negative charge on the phosphonate which opens an active site for nucleophilic attack and stabilize the leaving group. They found that the La^{3+} ions were able to catalyze the hydrolysis of the molecule by one, two, and three metal ions. They found that one ion was able to increase the rate of hydrolysis, but when two were used, the rate quadrupled. The very interesting finding was that they were able to observe a third ion being used in the hydrolysis, which at that point was novel. The reason why lanthanide ions show this ability is because of their charge of +3. This allows them to form unique complexes and also why they are less soluble, but effective at catalyzing reactions.

Alkaline metal ions were studied for an effect on phosphodiester hydrolysis at Drew University in Dr. Cassano's lab using new model systems[22]. This study used thymidine-5'-*p*-nitrophenyl phosphate, as their phosphodiester, and Mg^{2+} and Ca^{2+} as the alkaline earth metals. The results of this study showed that alkaline earth metal ions can catalyze the hydrolysis of phosphodiester bonds with both one and two metal ions. Ca^{2+} showed to catalyze the cleavage of the phosphodiester, thymidine-5'-*p*-nitrophenyl phosphate, by 900-fold and Mg^{2+} was seen to catalyze the cleavage by 1800-fold. This research has shown that there are different levels of

catalysis depending on the amount of metal ions present in the mechanism. One metal ion will catalyze the reaction; however, if there is another metal ion involved, the reaction rate will be further increased.

There were two key parts found for nucleophilic activation for phosphodiester reactions[5]. The first aspect is the position of the nucleophile, the nucleophile is more likely to attack the phosphodiester if it is closer to the phosphoryl group. The correct position could theoretically be obtained from a metal ion coordinating with the oxygens in the phosphoryl group as well as a hydroxide. The second aspect is increasing the nucleophilicity; the more nucleophilic the nucleophile, the more likely it will be to attack and increase the rate. A metal ion can activate a water nucleophile by aiding the loss of a proton due to the metal's Lewis acidity. The resulting hydroxide formation will, in, turn increase the nucleophilicity. Having to overcome the electrostatic repulsion between the negative phosphoryl group and the negatively charged nucleophile was found to not be as important for the rate of reaction. Looking at the different ways in which a nucleophile can be activated for nucleophilic attack explains why metal ions should be able to catalyze the hydrolysis reaction of these molecules.

1.6 Esterase Model Systems

Research of interest regarding phosphodiester cleavage is the effect of metal ion catalysis on molecules without a negative charge. This research is of importance because it shows how metal ions are able to interact with molecules that exhibit a partially negative charge, such as the carbonyl oxygen on esters. The interactions between metal ions and carbonyl oxygens can then be compared to the interaction between metal ions and the negative charge on the non-bridging oxygen on phosphodiesters, to see how important the negative charge is for metal ion catalysis. To

study this, we will look into molecules that have no negative charge, and see the effect metal ions have on catalyzing a hydrolysis reaction. Although there is carboxypeptidase, there are still not as many enzymes using metal ion hydrolysis for non-phosphodiesters as there are for phosphodiesters found in nature; this research could explain why this is in fact the case.

One way to study metal ion catalyzed hydrolysis with esters were by using hydrophobic complexes; these complexes simulate the inside of the enzyme binding pocket, a hydrophobic pocket[23]. They were looking at several different *p*-nitrophenyl carboxylates with the particular hydrophobic complex, *N*-methylhydroxamic acid. *N*-methylhydroxamic acid was found to efficiently catalyze the *p*-nitrophenyl carboxylates. The following study was using Cu^{2+} ions to test for catalytic ability with *N*-methylhydroxamic acid. They found that the Cu^{2+} ions were able to hydrolyze the carboxylates, which indicates that model systems can be used to study the catalytic ability of enzymes that can hydrolyze esters.

Some esters that are found in nature are amino acid esters and a study has been done to test the effect that heavy metal ions have on hydrolyzing amino acid esters[24]. They used a model system to study how metal ions are used in the activation of enzyme catalyzed hydrolysis reactions in nature. They worked primarily with Co^{2+}, but also looked at Cu^{2+}, Mn^{2+}, Ca^{2+}, and Mg^{2+}. What they found was that Co^{2+} was able to increase the rate of hydrolysis for glycine ethyl ester and had a Michaelis-Menten like fit for the rate. They also found that the hydrolysis rates increase with an increase in hydroxide concentration, which makes sense due to hydroxide being the nucleophile in hydrolysis reactions. It was also found that cobalt was able to catalyze the hydrolysis of many different amino acid esters, the largest effect was with the amino acid, glycine. The studies with the other divalent metal ions were not done as in depth as the cobalt ions, but what they found was that the rates of hydrolysis increased with the metals ability to incorporate into a complex. Calcium

had little to no effect on the hydrolysis of glycine ethyl ester, due to it forming unstable complexes. This study shows that metal ions can be used to catalyze hydrolysis reactions with esters and that model systems can be used in this manner to study metalloenzyme action, with several different metal ions.

1.7 p-Nitrophenyl Acetate

The molecule, *p*-nitrophenyl acetate (*p*NPA) is an ester with the leaving group *p*-nitrophenol (Figure 14). This molecule can be used to study how metal ions interact with partial negative carbonyl oxygens. Although it is not a phosphate ester, it can still be used to study the importance of the negatively charged non-bridging oxygens in phosphodiesters in metal ion catalysis. The *p*NPA is still an ester and therefore there is both a bridging oxygen and a non-bridging oxygen, the carbonyl. Another reason why *p*NPA has such a good leaving group is because the electron-withdrawing group, NO_2, is in the para position, leading to *p*-nitrophenol being a good leaving group[25]. Due to the electronegative nature of oxygens, the oxygens in *p*NPA will have partial negatives, and we are curious to see if these partially negative oxygens will be able to interact with metal ions, the same way metal ions interact with the non-bridging oxygens in phosphodiesters.

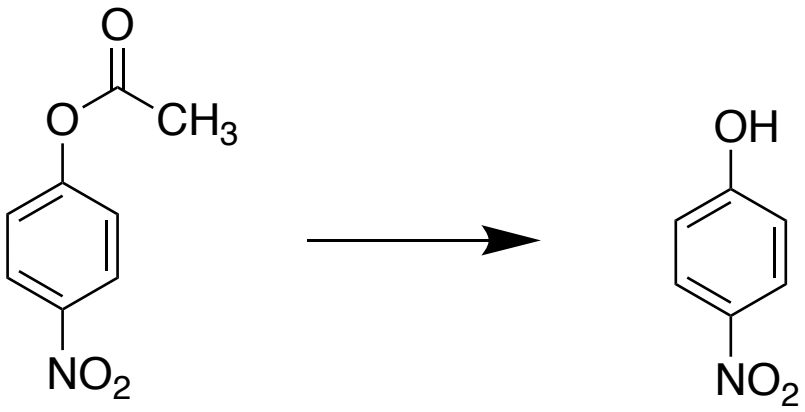


Figure 14 *p*-nitrophenyl acetate and its leaving group, *p*-nitrophenol. The structure of *p*-nitrophenyl acetate (*p*NPA). It contains an ester, and its leaving group is *p*-nitrophenol when the ester gets hydrolyzed.

It is important to understand the mechanism of hydrolysis of *p*NPA before experimenting and understanding catalytic effects of metal ions. One study done at University of Wisconsin used kinetic isotope effects to determine the transition state structure for *p*NPA hydrolysis[26]. More specifically, they measured the rate of hydrolysis of different nucleophiles in an aqueous and chloroform solution. The chloroform and aqueous layers were used to test the acyl transfers between *p*NPA and several different nucleophiles, to form *p*-nitrophenolate. The *p*-nitrophenol needs to be deprotonated to *p*-nitrophenolate in order for the reaction to occur. Chloroform is used because *p*-nitrophenolate is equally partitioned between the aqueous and chloroform layers. At the higher pH's that are needed for *p*-nitrophenolate to form, competitive aqueous hydroxide would hydrolyze the *p*NPA instead of an acyl transfer with the nucleophile. The reactions were observed in the buffer. They discovered that because there was such a large difference in the pKa values of the anionic nucleophiles and the leaving group, that any kind of tetrahedral intermediate would result in completely forming into product. This tells us that *p*NPA has such a good leaving group, that when it gets attacked by a hydroxide ion, it is more likely to proceed to the completed product, rather than reverting back to *p*NPA. This shows that the hydrolysis of *p*NPA should proceed pretty quickly, especially at higher pH levels. There is a tetrahedral transition state rather than a

tetrahedral intermediate, which means that the tetrahedral species is not present for long in solution. The tetrahedral species contains a negative charge which could interact with metal ions.

The same group of scientists from University of Wisconsin, identified more details about the transition states of *p*NPA[26]. Their isotope effect data showed that oxyanion nucleophiles result in substantial bond cleavage to the leaving group in the transition state. The main isotope they observed was the carbonyl oxygen. Other atoms looked at were the phenolic oxygen in the leaving group as well as the hydrogen in the acetyl group and the nitrogen in the leaving group. The kinetic isotope effect ratios being small showed that the bond to the leaving group is weakened too significantly for a tetrahedral intermediate to form; it is more likely that a tetrahedral transition state formed instead. When hydroxide was the nucleophile, the leaving group left the molecule a lot earlier in the transition state, compared to other nucleophiles. Also, the strength of the nucleophile was seen to make the most impact on transition state structure, not aromaticity and delocalization of the nucleophile. The final conclusions for this study was that the hydrolysis of *p*NPA with a strong nucleophile like hydroxide, results in a concerted mechanism (Figure 15). Even though hydroxide was shown to have some tetrahedral character, the dominant change in isotope effect resulted from the cleavage of the bond to the leaving group. This shows that because *p*NPA has such a good leaving group and hydroxide is such a good nucleophile, reacting *p*NPA in a basic aqueous solution will be favorable. Other work has agreed with the statement of *p*NPA hydrolyzing in a concerted mechanism due to its very good leaving group[25]. They computed the kinetic isotope effect (KIE) using the Bigeleisen – Mayer theory for the *p*NPA and hydroxide hydrolysis model. They were unable to find a tetrahedral intermediate nor an elimination transition state showing that a potential tetrahedral intermediate would be instable. The KIE for the leaving group oxygen was found to be inverse at 0.9982, and due to that being low, shows that the leaving

group oxygen and the carbon bond plays a very little role in the reaction coordinate, which indicates a concerted mechanism.

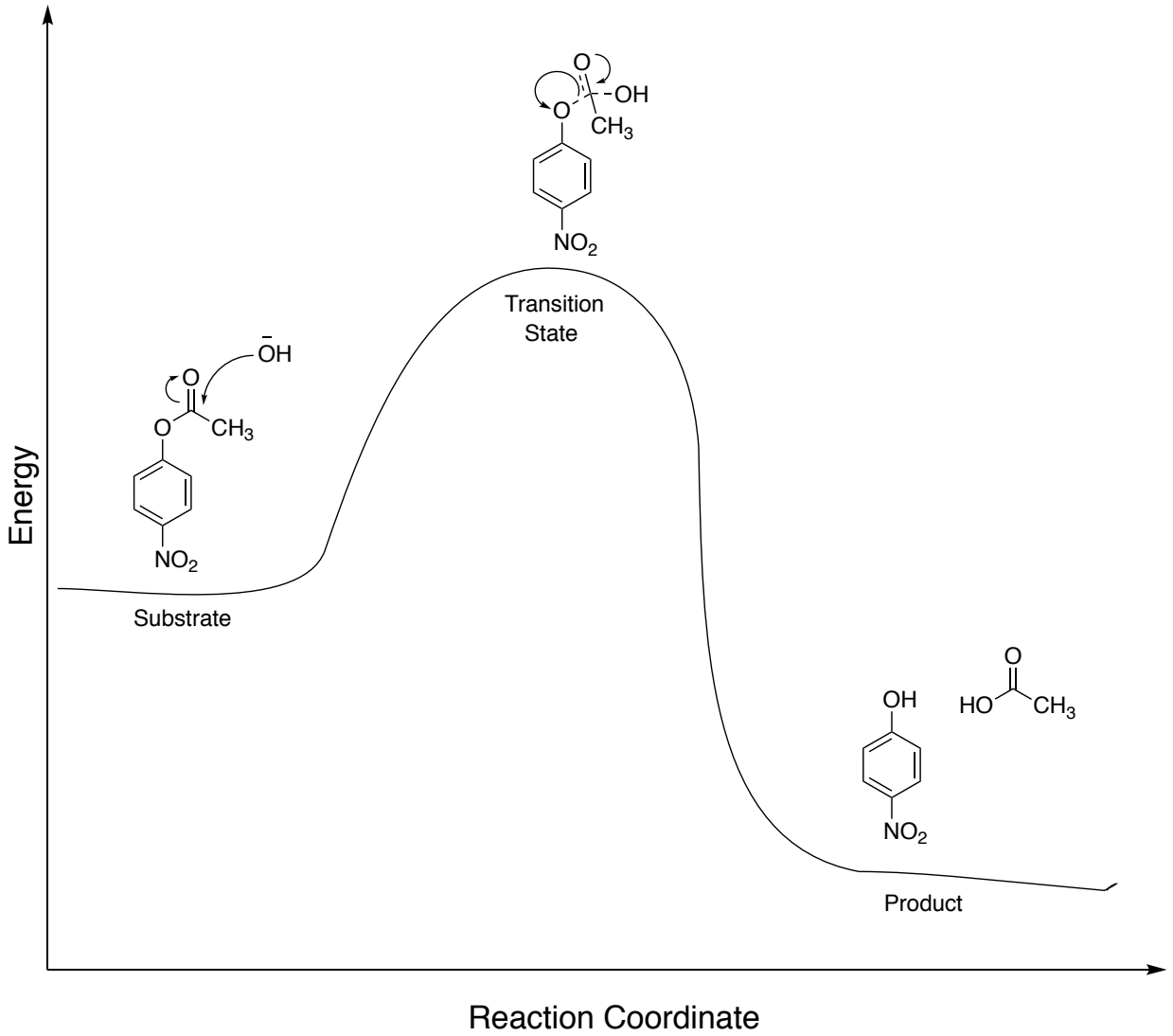


Figure 15 Reaction coordinate for the hydrolysis of *p*-nitrophenyl acetate with hydroxide as a nucleophile. As found through previous work, the hydrolysis of *p*NPA with a hydroxide nucleophile undergoes a concerted mechanism[26]. In the reaction coordinate, this is shown with the tetrahedral species only existing as a transition state rather than an intermediate. The dotted lines on the transition state is to show that the bond formation and bond breakage is occurring at relatively the same time.

Based off of previous work, there are a few proposed mechanisms for metal ion catalyzed hydrolysis of *p*NPA (Figure 16). The first is that the metal ion can coordinate a hydroxide attack

on the carbonyl (Figure 16A). The second way is that the metal ion can coordinate with the carbonyl oxygen which will polarize the bond, making the carbonyl carbon more partially positive and therefore a better electrophile for a hydroxide ion nucleophile (Figure 16B). A combination of the two mechanisms is also plausible, where the metal ion coordinates with both the nucleophilic hydroxide ion and the partially negative carbonyl oxygen. Two additional advantages in working with *p*NPA are its nontoxicity and the ability to monitor the product, *p*-nitrophenol (*p*-nitrophenolate when pH >7.15[27]) through UV-Vis spectroscopy[28].

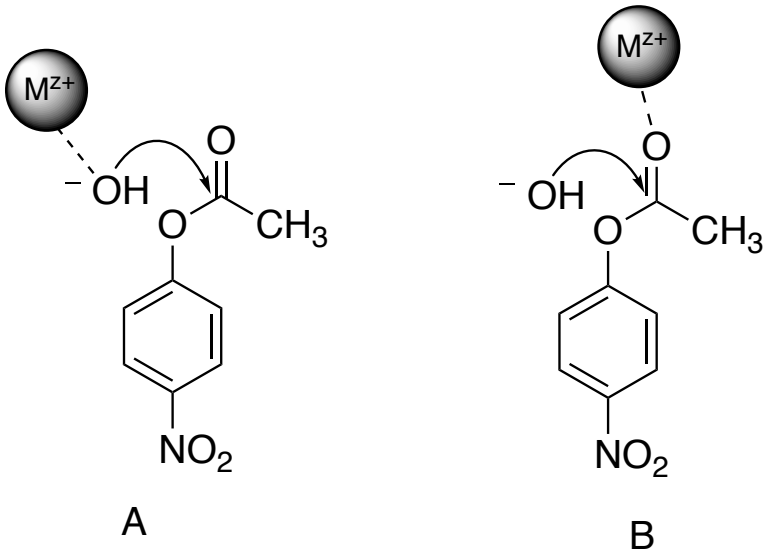


Figure 16 Proposed mechanisms of metal catalyzed hydroxide attack on *p*NPA based off previous work. *p*NPA contains an acetyl group that has a partially negative carbonyl oxygen. (A) Ca^{2+} ions could coordinate a hydroxide ion to attack the carbonyl, hydrolyzing the reaction. (B) Ca^{2+} ions could also interact with the partially negative carbonyl oxygen, polarizing it, which will make the carbonyl carbon more partially positive and more likely to be a target for nucleophilic attack. A combination of the two could also happen which would catalyze hydrolysis of *p*NPA.

Metallomicelles allow for an environment for metal ion catalyzed hydrolysis of esters to occur in an aqueous solution. There was a study done on the hydrolysis of p-nitrophenyl picolinate (*p*NPP) and p-nitrophenyl acetate (*p*NPA) (Figure 17) using Cu(II) and Zn(II) complexes dissolved in the cationic surfactant hexadecyltrimethylammonium bromide (CTAB) micellar solutions (Figure 18)[29]. The micellar solutions were used because of the low solubility of the metal ion

complexes in water. They found that adding just the CTAB comicelle, there was no significant increase in rate for both *p*NPP and *p*NPA. However, when they added Cu(II) and Zn(II) complexes to the solution there was a 22-fold and 54-fold increase in rate, respectively, for *p*NPA and a 3580-fold and 6847-fold, respectively, for *p*NPP. *P*NPP has a nitrogen in the picolinate group and *p*NPA has an uncharged acetyl group. They both were able to be catalyzed by divalent metals, but because of the nitrogen in *p*NPP that could interact with the metal complex, the increase in rate was a lot more significant. It was found that *p*NPA does not bind well to the binary metal complex, therefore the catalysis is thought to be done by intermolecular catalysis, compared to intramolecular catalysis, like with *p*NPP. This means that the system that we are using with calcium ions in solution is possible for catalysis to occur but it is expected to be less compared to charged molecules. Our system is expected to be an intermolecular mechanism for catalysis, where the hydroxide will be activated and attack the *p*NPA. This could have been what happened in this experiment, so it gives a good reason to look into it for ours.

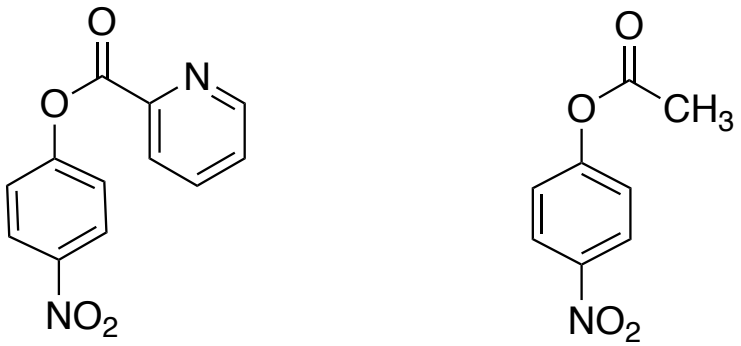


Figure 17 Structures of *p*-nitrophenyl picolinate compared to *p*-nitrophenyl acetate[28]. The structures are very similar, the only difference being that *p*-nitrophenyl picolinate has a picolinate group and *p*-nitrophenyl acetate has an acetate group.

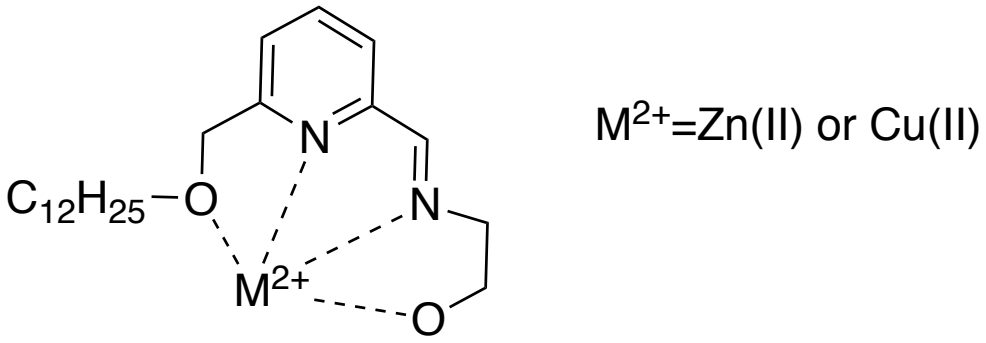


Figure 18 The metal complexes used to test the catalysis of *p*NPA and *p*NPP[29]. These metal complexes were used to see if they could catalyze the hydrolysis of *p*NPA and *p*NPP. They tested with the same ligand but with Zn(II) and Cu(II).

A similar study to the Zn(II) and Cu(II) complexes in CTAB micellar solutions was done, but this time, Cu(II) and Zn(II) Schiff base complexes were used (Figure 19). The hydrolysis of both *p*NPA and *p*NPP were investigated with the Schiff base complexes of both metal ions, in both Brij35 micellar solutions and nonmicellar solutions. The two complexes had their distinct differences, the Zn(II) complex was able to coordinate with a water molecule as well where the Cu(II) complex did not, which could lead to different mechanisms of hydrolysis (Figure 20). It was found that the Cu(II) complex was able to catalyze the hydrolysis of *p*NPP more than the Zn(II) complex could, 80-fold and 53-fold respectively (Table 1). However, with *p*NPA, the results were the opposite, the Zn(II) complex was able to catalyze the hydrolysis reaction better than the Cu(II) complex; the Zn(II) complex increased the rates by 9 fold where the Cu(II) complex barely had an effect on hydrolysis rates. When the substrates were exposed to Brij35 micellar solution, hydrolysis rates went down slightly, approximately 40% for both *p*NPP and *p*NPA, but when the complexes were added the rates of hydrolysis went up. With *p*NPP, the micellar solutions with complexes increased hydrolysis rates even further than without the micellar solutions, the Cu(II) complex with Brij35 increased hydrolysis rates by 100 fold when compared to just Brij35 and no metal complex. For the Zn(II) complex, this increase was 61 fold. The increase in hydrolysis rates

between buffer only and metal complexes with Brij35 micellar solution were lower than the metal complexes in solution without Brij35, Cu(II) being 59-fold and Zn(II) being 36-fold for *p*NPP; which showed that adding this micellar solution does not increase overall rates more than standard buffer with this particular metal complex. For *p*NPA, the micellar solutions led to even less catalysis when used in conjunction with the metal complexes. The Zn(II) complex led to a 4-fold increase and the Cu(II) complex increased hydrolysis rates negligibly.

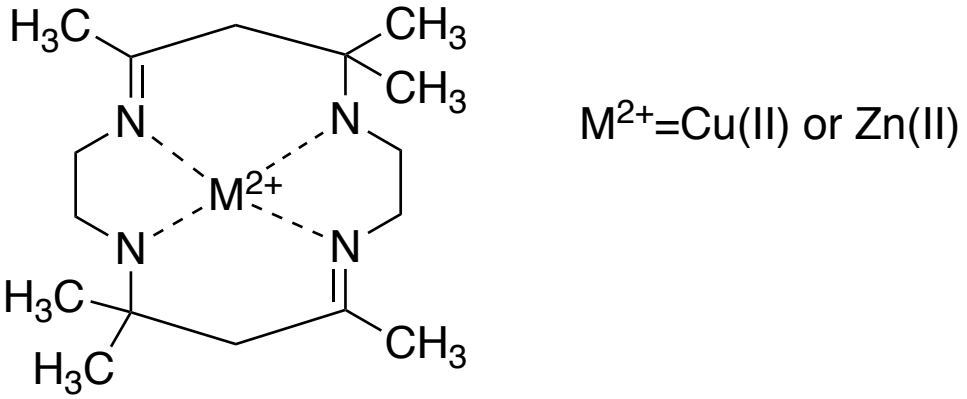


Figure 19 The ligand used to study metal complex catalysis for the hydrolysis of *p*-nitrophenyl acetate and *p*-nitrophenyl picolinate[28]. This complex was used to coordinate with either Cu(II) or Zn(II) to catalyze the hydrolysis of *p*-nitrophenyl acetate and *p*-nitrophenyl picolinate.

Table 1 First order rate constants for the hydrolysis of *p*NPP and *p*NPA in the presence of Zn(II) and Cu(II) complexes and Brij35 micellar solution[28].

System	10^3 k_{obs} (*p*NPP)/s^{-1}	10^5 k_{obs} (*p*NPA)/s^{-1}
Buffer	0.0442	1.82
Buffer + CuL	3.52	1.89
Buffer + ZnL-H_2O	2.35	16.8
Buffer + Brij35	0.0261	1.11
Buffer + Brij35 + CuL	2.61	1.13
Buffer + Brij35 + ZnL-H_2O	1.60	4.47

Buffer = 0.1 mol dm^{-3} Tris-HNO_3, ionic strength = 0.1 (KNO_3); [Brij35] = 5.00 x 10^{-3} mol dm^{-3}; [CuL] = [ZnL-H_2O] = 4.10 x 10^{-3} mol dm^{-3}; [pNPP] = [pNPA] = 5.00 x 10^{-5} mol dm^{-3}.

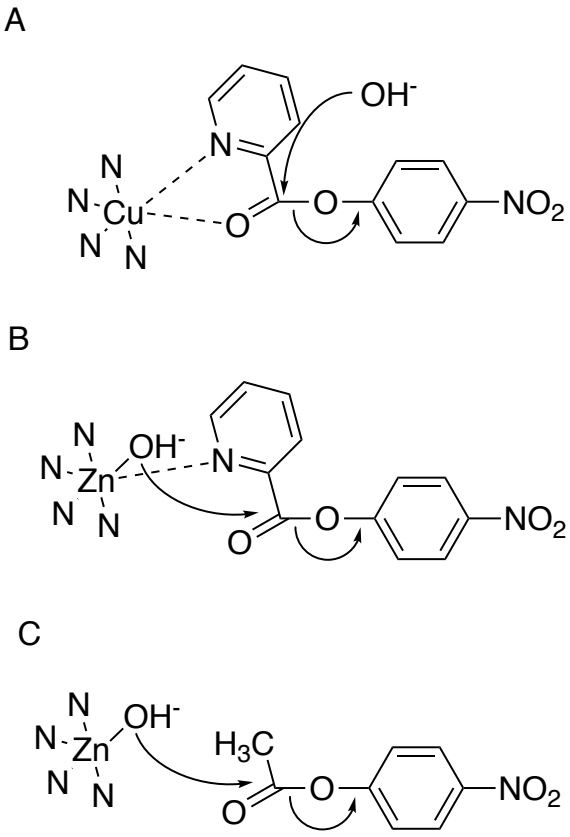


Figure 20 The hydrolysis of *p*NPP and *p*NPA with metal complexes[29]. The mechanisms in which the Schiff base complexes of Cu(II) and Zn(II) hydrolyze *p*NPP and *p*NPA. (A) The Cu(II) ion coordinates with the carbonyl oxygen as well as the nitrogen in the picolinate group of *p*NPP which polarizes the carbonyl, making it more electrophilic for the nucleophilic attack of a hydroxide ion. (B) The Zn(II) ion is able to coordinate with the nitrogen in the picolinate group of *p*NPP to polarize the carbonyl to make it more electrophilic, as well as make the metal bound hydroxide ion occur intramolecularly instead of intermolecularly. (C) The metal complex is not able to bind to *p*NPA, so therefore the catalysis occurs via a hydroxide bound Zn(II) ion complex acting as a nucleophile.

The reason why *p*NPP was believed to be catalyzed more for this complex was the same reason as described before[28]. The nitrogen in the picolinate group of the substrate is able to interact with the metal ion core of the Schiff base ligand, leading to a greater catalysis. The Cu(II) complex, is able to interact with both the nitrogen in the picolinate group and the carbonyl oxygen of *p*NPP which led to the large increase in hydrolysis rates (Figure 20A). The Cu(II) complex however did not increase *p*NPA hydrolysis by a significant amount; this was because the coordination between the Cu(II) ion and the carbonyl oxygen on *p*NPA is really low, especially compared to the nitrogen

on *p*NPP. With the Zn(II) complex, both *p*NPP and *p*NPA were hydrolyzed, *p*NPP more than *p*NPA though. This was still due to the fact that *p*NPP has the picolinate group that not only contains a nitrogen that can coordinate with the complex metal, but it also is an electron-drawing group which would stabilize the negative charge on the transition state where the methyl group on *p*NPA is electron-donating and will destabilize the negative charge on the transition state (Figure 20B). The reason the Zn(II) complex was able to catalyze *p*NPA was because the complex was able to coordinate with a water molecule which was used as the nucleophile (Figure 20C). The water molecule was activated by the Zn(II) complex which made it more nucleophilic, which led to catalyzing the hydrolysis of both *p*NPP and *p*NPA.

1.8 p-Nitroacetanilide

In order to understand how *p*NPA is interacting with the metal ion, mechanistically, *p*NAA will be observed (Figure 21). *p*NPA and *p*NAA are very similar molecules, the only difference being the leaving groups, *p*NPA's being *p*-nitrophenol and *p*NAA's being *p*-nitroaniline. The reason why we believe that there may be a difference in metal ion catalysis between these two molecules is because of the alcohol group on *p*-nitrophenyl makes it a better leaving group than *p*-nitroaniline because *p*-nitroaniline has an amino group instead of an alcohol group. The pKa of *p*-nitrophenol is 7.15[27] and the pKa of *p*-nitroaniline is 18.9[30], this indicates that *p*-nitrophenolate is more stable with a negative ion, and therefore is a better leaving group, than *p*-nitroaniline.

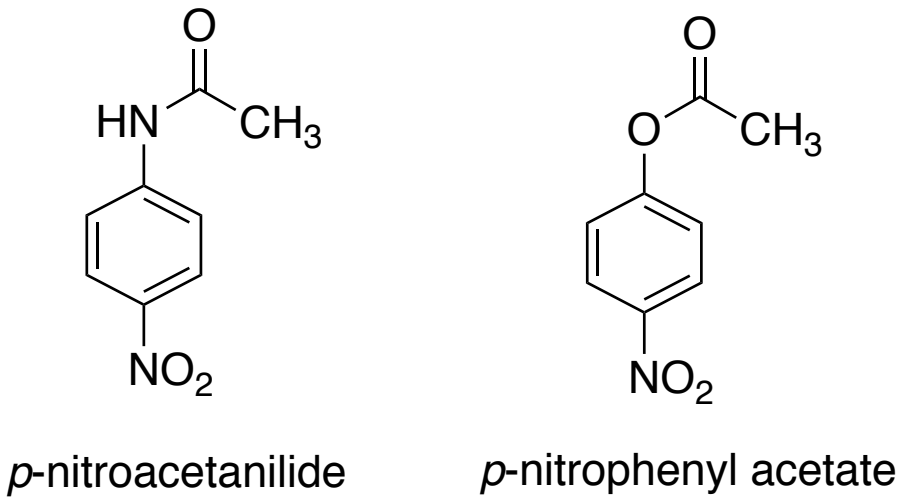


Figure 21 Structure of the molecules, *p*-nitroacetanilide and *p*-nitrophenyl acetate. The structure of *p*-nitroacetanilide is very similar to *p*-nitrophenyl acetate. The only difference being that *p*-nitroacetanilide has an amine in the leaving group, where *p*-nitrophenyl acetate has an alcohol instead.

Esterases are enzymes found in nature that are able to catalyze the hydrolysis of esters, such as *p*NPA. Research has been done on the mechanism for the esterase antibody, 43C9[31]. Usually this antibody is used in its function of cleaving anilides, but it has been found that it can also cleave aryl esters, like *p*NPA. The studies looked at both amides and esters to see how the mechanisms of the two differed. One ester they looked at was *p*NPA and one amide they worked with was *p*-nitroacetanilide. They calculated the average binding energies for the ground state and the transition state to find the mechanisms. They found through computational studies that the ester hydrolysis was concerted because there was only one transition state and the amide hydrolysis was stepwise because there were two transition states. This is interesting because these two molecules are almost identical, only differing from one being an amide and the other being an ester. This shows that the bridging oxygen in *p*NPA is responsible in making *p*-nitrophenol such a good leaving group in hydrolysis reactions, which is not surprising due to *p*-nitrophenol being far more electronegative than *p*-nitroaniline. Further studies in this work was to determine the way in which the nucleophile attacked the molecules[31]. Through gas chromatography and molecular dynamics

simulations, it is proposed that direct hydroxide attack is the most plausible mechanism for antibody 43C9. The most likely way this hydroxide is formed in the antibody is by a histidine deprotonating a water molecule, forming hydroxide ion. Even though this mechanism does not utilize a metal ion, this is most likely the way in which esters are hydrolyzed with enzymes in nature.

Due to *p*NPA's leaving group being so great, metal ions may not be needed too much to stabilize the negative charge of the oxygen in the transition state, because of the concerted mechanism[31]. However, *p*NAA's hydrolysis is stepwise[31]; therefore a metal ion could be used to stabilize the negative charge on the oxygen in the intermediate, speeding up the reaction. This will allow us to understand how metal ions catalyze reactions with good leaving groups versus bad leaving groups. Another thing that studying *p*NAA in addition to *p*NPA is how metal ions interact with *p*NPA and carbonyls in general. The way this is concluded is by seeing the difference in increased catalysis of metal ions for both molecules; if they both increase by the same amount, the catalysis is most likely due to a metal ion activating a water molecule. If the *p*NAA rates were increased more than the *p*NPA rates were increased, it could be because the metal ions are interacting with the carbonyl because of *p*NAA's stepwise mechanism and *p*NPA's concerted mechanism.

A study was done on the hydrolysis of *p*-nitroacetanilide, to understand the mechanism, because the reaction was faster than previously predicted, because their previous studies showed that rates of hydrolysis are independent of the substituents for the ring substituents that they previously looked at (Figure 23)[32]. The hydrolysis rates were found to be more dependent on the hydroxide concentration, compared to other anilides studied. Also, this led them to believe that there is a different mechanism for anilide hydrolysis when an electron-withdrawing group is

present on the phenyl, like NO_2. The new mechanism is proposed to expose a negatively charged anilide ion rather than a neutral aniline molecule, which is related to *p*NPA hydrolysis. Through a log k_{corr} vs log [$^{-}$OH] plot, it was found that there were two hydroxides involved in the hydrolysis of *p*NAA, as shown by the slope being 1.9. They found that at pH values lower than 13, for *p*NAA, the rate for hydroxide ions is second order and at pH values higher than 13, the rate for hydroxide ion becomes less than second order. To accommodate for the two hydroxide ions used in the mechanism, after the hydrolysis, there is likely a negative charge on one of the oxygens and on the amide. There were two possible mechanisms envisioned for how this would be possible (Figure 22). One mechanism uses a hydroxide ion as a base and the reaction is hydrolyzed via an acid base reaction (Figure 22a). The other mechanism, starts with the anilide being ionized before breaking down into the products (Figure 22b). It would be interesting to note if there are multiple hydroxides used in the hydrolysis reaction of *p*NAA. This would allow multiple metal ions to interact with all of the different hydroxide ions, and could potentially catalyze the reaction. It may seem unlikely for *p*-nitroacetanilide to form an intermediate with an overall charge of -2, but it was found that there are two possible intermediates formed one with a net charge of -1 (Figure 22A) and one with a net charge of -2 (Figure 22B)[33]. Having the net charge of -1 is more likely to occur, but the mechanism with a -2 charge is still able to occur.

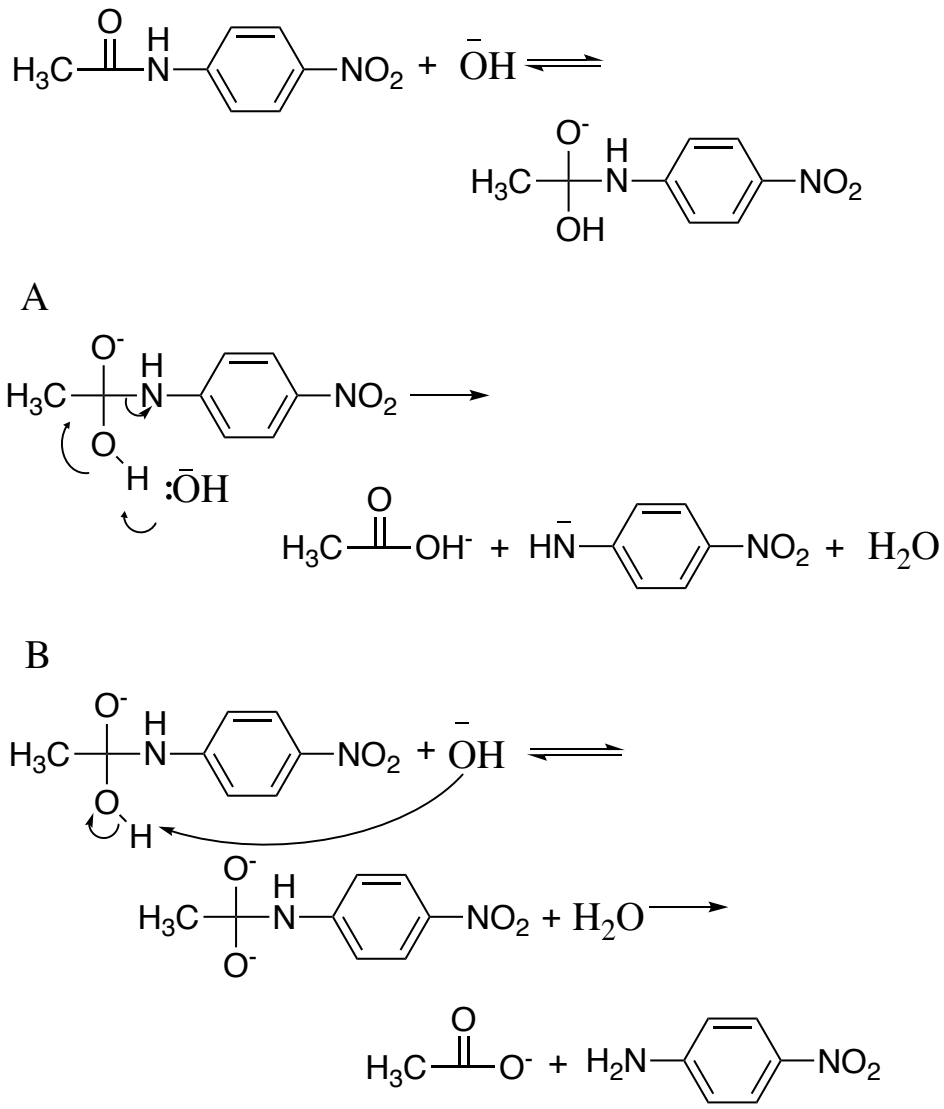


Figure 22 Two proposed mechanisms of the hydrolysis of *p*-nitroacetanilide. Modified from Bender et.al.[32]. Two different mechanisms were proposed for how two hydroxides were used to hydrolyze the molecule *p*-nitroacetanilide at lower pH values. Both mechanisms start off with an initial attack of hydroxide on the carbonyl carbon. (A) A hydroxide ion is used as a base for a general acid base catalysis and hydrolyzes the reaction in one step. (B) A hydroxide ion deprotonates the hydrogen on the oxygen to form an intermediate with a charge of (-2),because there are two hydroxides involved, and water hydrolyzes the reaction.

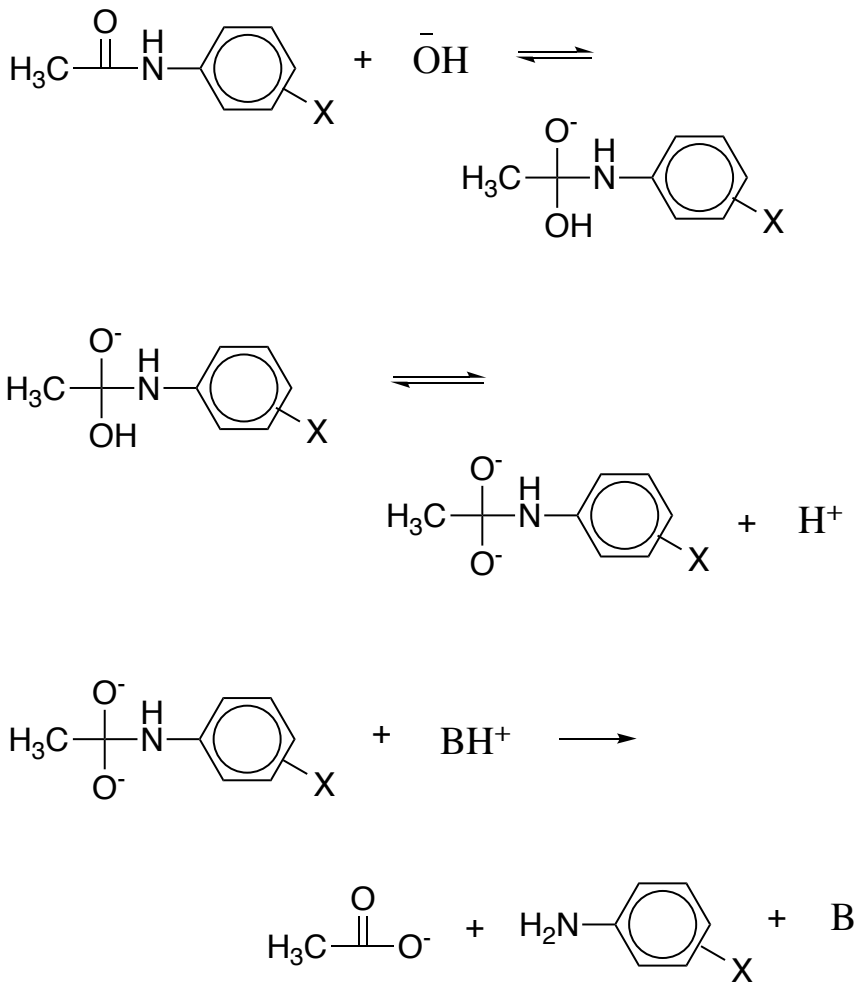


Figure 23 Proposed mechanism of anilides with one hydroxide present[32]. The previous anilides studied were found to utilize only one hydroxide ion. This hydroxide ion is used as a nucleophile to attack the carbonyl carbon to form the tetrahedral intermediate.

The reason why the hydrolysis of *p*NPA and *p*NAA could be catalyzed differently by metal ions is because the two molecules' hydrolysis reactions have different rate limiting steps[34]. The rate limiting step for esters is the concerted bond formation between the nucleophile and the carbonyl carbon, and breaking of the bond between the carbonyl carbon and nitrophenyl leaving group. The rate limiting step for amides is solely the C—N bond breaking (Figure 24) (Figure 25). A study done to compare amide and ester hydrolysis used copper containing micelles with a variety of different esters and amides. The hydrophobic ligand, *N,N,N'*-trimethyl-*N'*-hexadecylethylenediamine was used in conjunction with cupric chloride to create the copper

containing micelles. The belief was that the copper could coordinate with the carboxylic acid group on an ester to bring the ester closer to a metal-bound hydroxide, thus catalyzing the reaction. With the esters, they found with the absence of an acid group, such as *p*NPA the reaction only gets catalyzed by a smaller amount, around 5 fold, compared to 2-acetoxy-5-nitrobenzoic acid, which contains a carboxylic acid in the ortho position, was catalyzed by 7100- fold. This was because the rate limiting step is bond formation, and the position of the substrate in the micelle is kinetically important because it needs to be close to the metal-bound hydroxide. For the amide, they found that the hydrolysis was caused by a copper-bound hydroxide and not by a hydroxide ion from the bulk solution. Due to the rate determining step being bond breaking rather than bond formation, the orientation of the substrate in the micelle is not kinetically important. This study showed that catalysis differs between amides and esters due to the different rate limiting steps.

It is widely known that amides have to go through the rate limiting step of breaking down the tetrahedral intermediate in order to be hydrolyzed[35]. The effects of zinc ion were tested for the hydrolysis of amides and how the zinc ion catalyzes the reaction specifically. What they found was that the zinc interacts with a water molecule which can then be used as a base or a nucleophile. They believe that a metal ion can significantly increase the rate of reaction of any amide as long as the metal allows two things: That the metal ion can bind to the amide in its tetrahedral intermediate, without interacting with the leaving group nitrogen (interaction with the leaving group nitrogen would inhibit required protonation) and that the metal ion cannot alter the amide's shape to the point that the reaction cannot continue. This study indicates that it is highly plausible that calcium is able to catalyze the hydrolysis of *p*NAA.

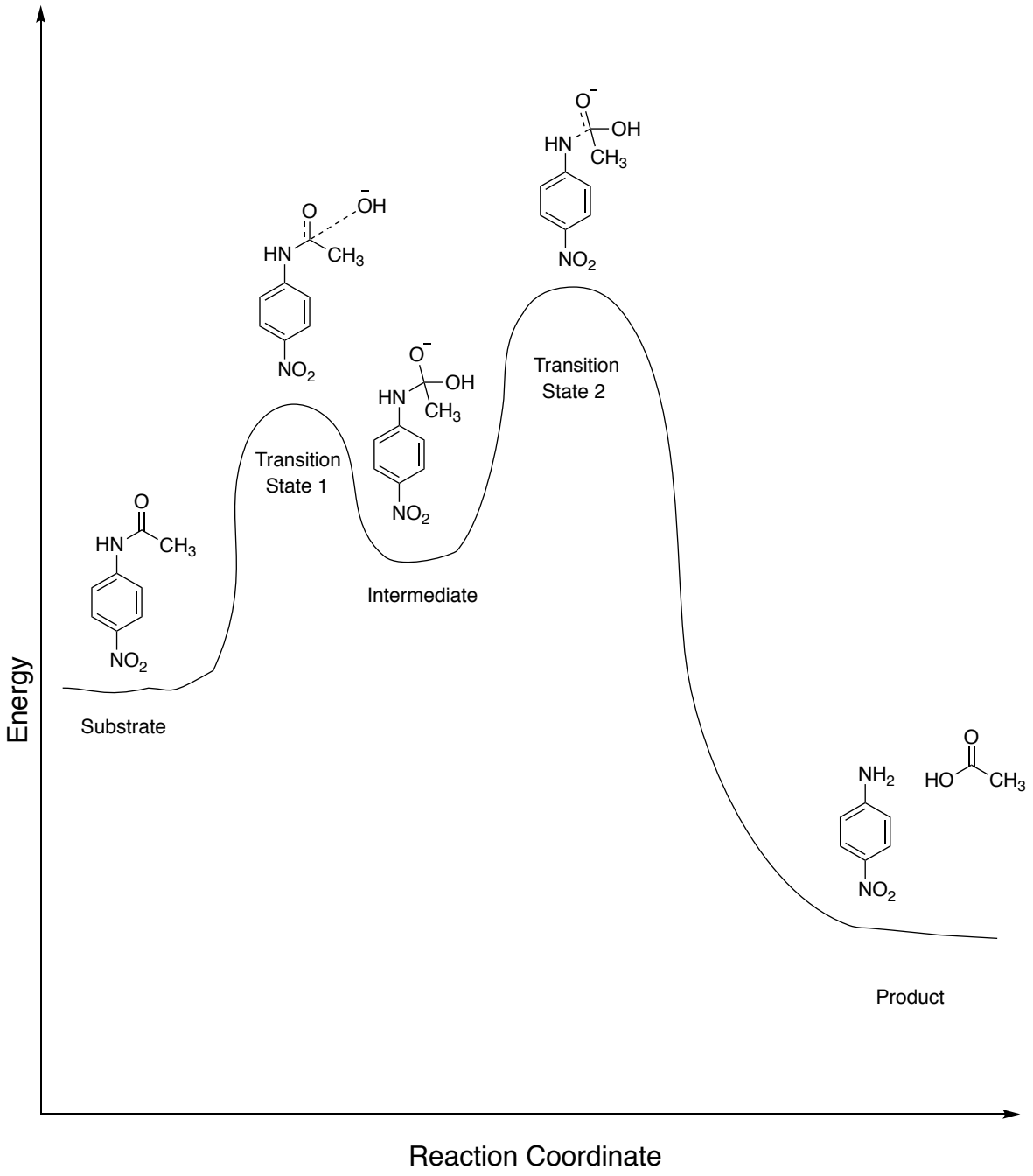
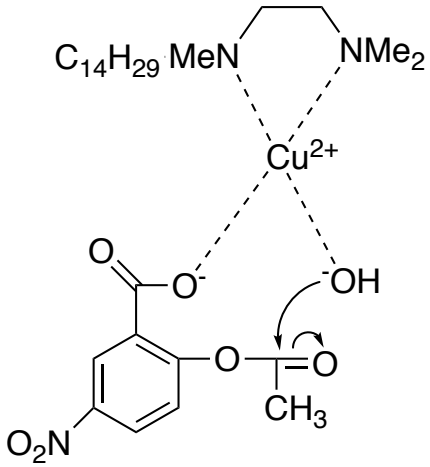
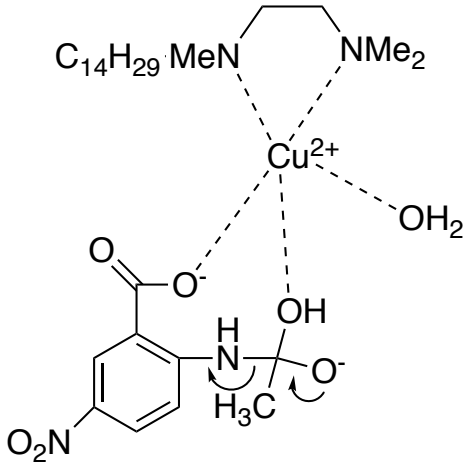


Figure 24 Reaction coordinate of the hydrolysis of *p*-nitroacetanilide[34]. The hydrolysis of *p*-nitroacetanilide is believed to be done in a stepwise reaction, where there is a tetrahedral intermediate present. The rate-determining step is thought to be that of transition state 2 where the C—N bond is breaking.



Rate-determining transition state for ester hydrolysis



Rate-determining transition state for amide hydrolysis

Figure 25 Rate-determining step for ester hydrolysis versus amide hydrolysis[34]. The rate-determining step for ester hydrolysis is the bond formation between the nucleophile and carbonyl carbon. The rate-determining step for amide hydrolysis is the C—N bond breaking.

There has been research done on phosphodiesters and non-charged esters on how to catalyze hydrolysis reactions. So far, metal ions were tested to see how they affect rates of hydrolysis reactions for both phosphodiesters and non-charged esters, and were found to generally increase the rate with both substrates. Previous research focused on using Ca^{2+} for catalyzing the

hydrolysis of phosphodiesters because there was no research in the community using Ca^{2+} as a catalyst for phosphodiester cleavage[3, 20]. The reason Ca^{2+} is used is because Ca^{2+} does not precipitate out of solution at higher pH's, like other metal ions do. The reason for the heightened pH's is so the role of OH^- as an external nucleophile can also be tested. Another reason for the heightened pH is to allow the reaction to proceed more quickly in order to have enough data in a shorter amount of time. However, there has yet to be research done on the effect of Ca^{2+} ions on the hydrolysis of non-charged esters. This would allow us to fill the empty calcium hole when it comes to non-charged ester hydrolysis. This research will also give us an understanding on how divalent metal ions, specifically Ca^{2+}, interact with carbonyls in model systems.

2.0 Methods

2.1 Sample Preparation of pNPA and pNAA

All of the samples that were prepared for kinetic analysis were 1 mL. This 1 mL of sample consisted of 50 mM CAPs Buffer at various pHs. The pHs used were 9.50, 10.45, 10.70, 10.90, and 11.00. Each sample contained a different amount of $CaCl_2$, to introduce the Ca^{2+} ion into solution. There were eight different Ca^{2+} concentrations used for each pH, they consisted of 0 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, and 333 mM. Each solution was normalized to an ionic strength of 1 using NaCl, to prevent an observed catalysis due to ionic strength rather than Ca^{2+} ion. The solution being tested contained 0.05 mM of *p*-nitrophenyl acetate (*p*NPA), because using Beer's law and the extinction coefficient of $\varepsilon_{405} = 18{,}500\ M^{-1}cm^{-1}$ [36] in water, it was shown that this concentration should have a max absorbance of 1. The pH values were also adjusted for by measuring the true pH after the addition of the CAPs, water, NaCl, and $CaCl_2$.

Due to the low solubility of *p*-nitroacetanilide (*p*NAA) in water, it had to be dissolved in DMSO. The samples contained 0.10 mM of *p*NAA, and due to the low amount of DMSO (10 μL in 1000 μL) in the final solution, it is considered negligible. This was tested with three different Ca^{2+} concentrations, at a pH of 11.00. The Ca^{2+} concentrations consisted of 0 mM, 150 mM, and 333 mM and were corrected with NaCl to normalize the ionic strength to 1. Using Beer's law and the extinction coefficient of $\varepsilon_{410} = 8{,}800\ M^{-1}cm^{-1}$ [37] in water, the max absorbance at 0.10 mM of *p*NAA would be 1. The maximum absorbance for nitroaniline is around 380 nm but that value has an overlap with the anilide substrate, so 410 is used instead.

2.2 UV-Vis Spectroscopy

The UV-Vis Spectrophotometers being used for these experiments were Cary 60s from Agilent Technologies. UV-spectroscopic studies from 200 nm to 500 nm were tested with 0.05 mM *p*NPA in 50 mM CAPs Buffer at pH 11.0 and 11.5. This initial scan was used to see if our *p*-nitrophenolate absorbance matches that of the literature'[28] (Figure 26). The scans done after were simple kinetics at the wavelength of 401.0 nm. This matches previous studies of *p*NPA hydrolysis where the product is usually tested around 400.0 nm. These reaction scans were 0.05 mM *p*NPA, 50 mM CAPs buffer at pHs of 9.50, 10.45, 10.70, 10.90, and 11.00, at each pH, 0, 50, 100, 150, 200, 250, 300 and 333 mM $CaCl_2$ were tested. The reactions ran until there were no longer product formation and because the reaction is strongly product favored, it is basically until 100% of the substrate has reacted. This was to ensure that the reaction ran long enough to determine the rates of product formation through the integrated 1st order rate equation fit lines explained later. During the reactions there were periodic scans using the kinetics program, the scans varied from 30 seconds to 6 seconds between each one, the higher the pH, the shorter the time between scans.

Due to the hydrolysis of *p*NAA being significantly slower than *p*NPA, only pH 11.00 was used for the spectroscopy studies. Also, only $CaCl_2$ concentrations of 0, 150, 333 mM were tested at pH 11.00 to indicate if metal ion catalysis occurs or not.

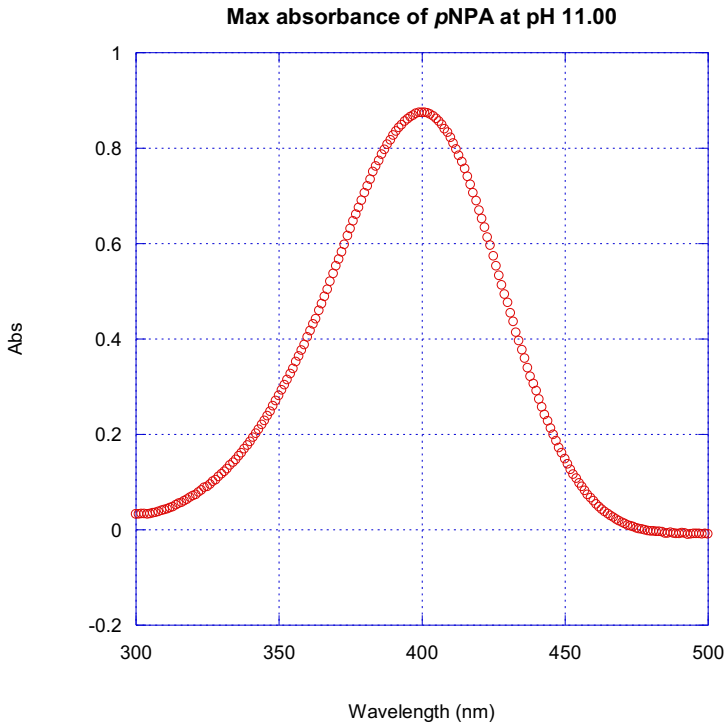


Figure 26 Max absorbance of *p*NPA's hydrolysis product, *p*-nitrophenol. Using UV-Vis Spectrophotometry, the hydrolysis product of *p*NPA, *p*-nitrophenol, at pH 11.00 was shown to have a λ_{max} of 401.0 nm.

2.3 Adjusting for [$^{-}$OH]

Due to $CaCl_2$ lowering the pH of the solutions when more were added, the [$^{-}$OH] had to be adjusted for before making the data plots. The way this was done was by making mock solutions of all the different reaction conditions that were tested, all the pH values and all of the $CaCl_2$ concentrations. These mock solutions were then tested for their pH with a pH electrode. These adjusted pH values were then used for future [$^{-}$OH] values.

2.4 Data Analysis using Kaleidagraph

The rates were found using Kaleidagraph and the absorbance values from the UV-Vis Spectrophotometers. The data received from the spectrophotometer studies for *p*NPA were graphed on to Kaleidagraph and an integrated 1st order rate equation applied to product was used as the general fit to find the rate of reaction; $[1]_t = [P]_\infty - Ae^{-kt}$. The rates of all of the pHs at the varying metal concentrations were plotted on a [$^-$OH] vs rate plot. This plot had a linear fit from Kaleidagraph and the slope was the pseudo first order rate constant. The pseudo first order rate constants at each of the [$CaCl_2$] were plotted against [Ca^{2+}]. This graph's general fit in Kaleidagraph used an equation for a straight line. The slope of the line will be the k_{obs} for the calcium induced catalysis. The reason that an integrated 1st order rate equation was ended up being used was because when the data was fit to multiple different models, whether it was rectangular hyperbola, straight line, quadratic function, or the 1st order rate equation, the fit that was the best, was used, in this case it was the integrated 1st order rate equation. Each of these models suggests a different mechanism and number of ions that the reaction could undergo.

Due to the studies of *p*NAA hydrolysis occurring too slowly, the reactions were unable to yield 100% product formation. Due to that, only the initial rates were found by using a linear fit.

3.0 Results

3.1 p-Nitrophenyl Acetate Kinetics

The reaction rate of *p*NPA was determined by measuring the appearance of the product, *p*-nitrophenol, in this case *p*-nitrophenolate because the phenol hydroxyl group is deprotonated at the pH values used for these reactions (9.50 and above compared to a pKa of 7.15[27]). The reactions ran at varying concentrations of $CaCl_2$: every 50 mM from 0 mM to 333 mM. Each $CaCl_2$

concentration were also ran in triplicate at the pH values: 9.50, 10.45, 10.70, 10.90, and 11.00. The reactions ran long enough to be able to determine where the product concentrations plateaued (Figure 27), which indicates that several half-lives occurred. Preliminary scanning kinetics were ran with a sample of 0.5 mM *p*NPA and 333 mM $CaCl_2$ at pH 11.5 to measure the λ_{max} of *p*-nitrophenolate. The λ_{max} was found to be 401.0 nm, and this value is supported by literature[28, 38], so thus, all of the following kinetic studies for *p*NPA was measured at this wavelength. As shown in the graph, the rate in reaction increases as the pH increases. Using an integrated 1st order equation for product accumulation in Kaleidagraph, the rate constants were found at each pH in the absence and presence of $CaCl_2$. The rates without Ca^{2+} for pH 9.50, 10.45, 10.70, 10.90, and 11.00 were 0.021 min^{-1}, 0.25 min^{-1}, 0.32 min^{-1}, 0.47 min^{-1}, and 0.67 min^{-1} respectively. The rate of the reaction increases as the pH was raised. The max absorbances do not line up in the Figure 27. The reason for this could be due to pipetting errors in the particular samples. However, this does not change the rates in the reaction because the reaction ran to completion. The reactant concentration is not used in the rate equations as the rate constants are independent of reactant concentration.

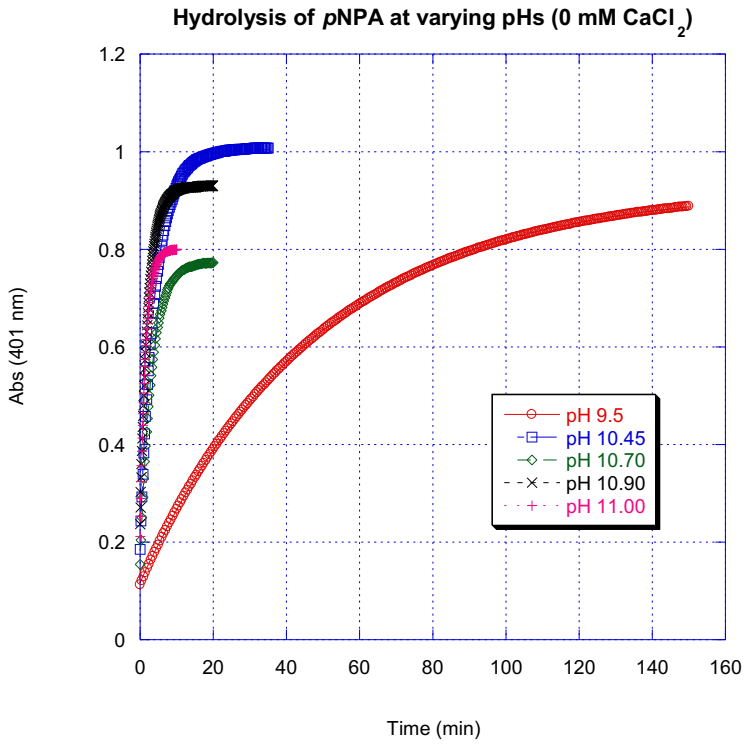
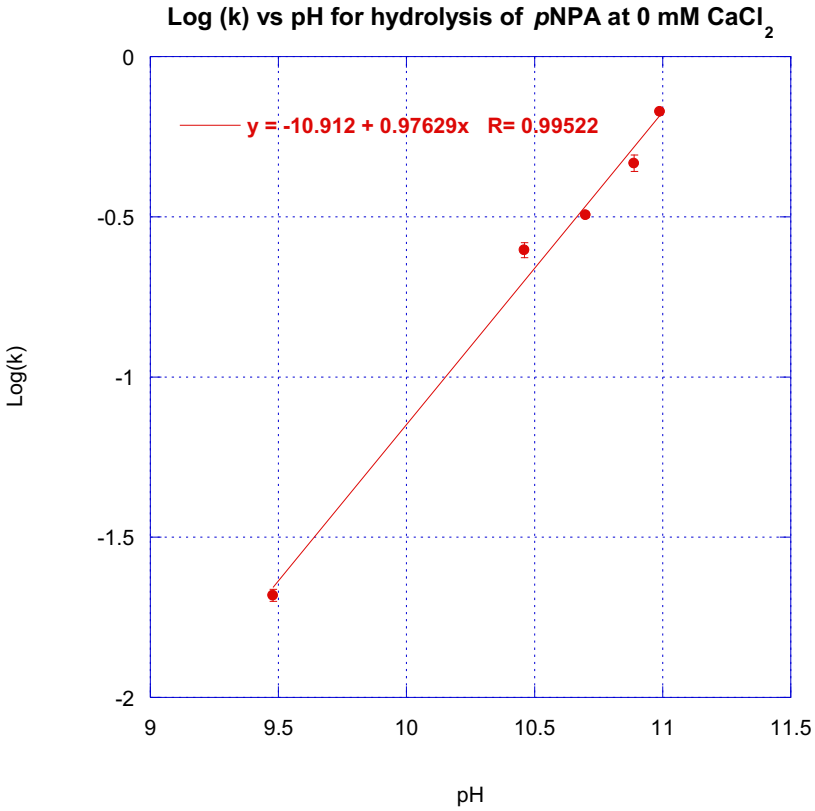


Figure 27 Hydrolysis of *p*NPA at varying pHs with 0 mM [$CaCl_2$]. These data were found using UV-Vis Spectrophotometry, measuring the appearance of the product, *p*-nitrophenolate at the absorbance, 401 nm. The reactions contained 50 mM of CAPs buffer, 0.05 mM of *p*NPA, 1000 mM NaCl at the varying pHs. The rate of hydrolysis increases as the pH is increased.

To determine the relationship between [$^-$OH] and the hydrolysis of *p*NPA, a log of rate versus log of [$^-$OH] plot was made (Figure 28). There were no $CaCl_2$ present in these data and were found using UV-vis spectrophotometry. This plot shows a linear fit, with a high R^2. This Figure was used to determine the number of $^-$OH molecules are involved in the mechanism. The slope of the line will determine this, and the slope is equal to 0.98 which is close to 1.



n = nth order with respect to $^{-}$OH

$$\log k = n \log[OH^-] + C$$

$$y = pH$$

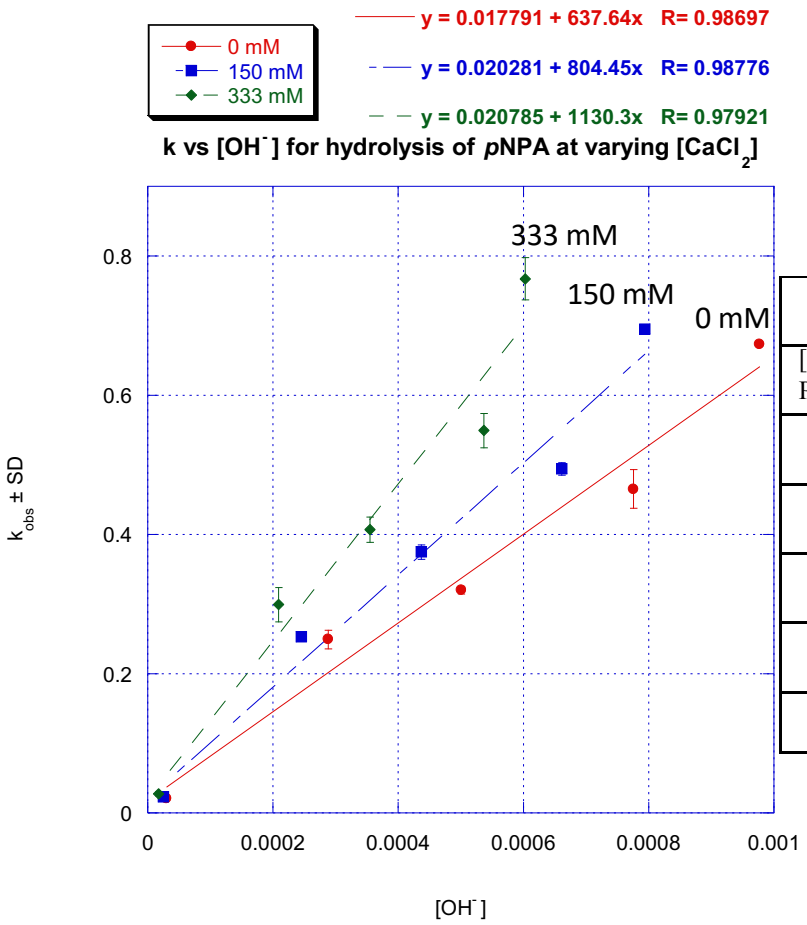
pH	StDev
9.48	0.0190
10.46	0.0236
10.70	0.0077
10.89	0.0257
10.99	0.0010

Figure 28 Log(k) versus pH for the hydrolysis of *p*NPA at 0 mM [$CaCl_2$]. The log of the rate at each of the different pHs were plotted against their pH. These rates were found using UV-vis spectrophotometry by measuring the appearance of the product, *p*-nitrophenolate. All of the reactions contained, 50 mM of CAPs buffer at various pHs, 0.05 mM *p*NPA, and 1 M NaCl. The slope of the plot is 0.98 which is close to 1, which indicates that the mechanism uses only one hydroxide ion.

Using these rate constants and the pHs that the reactions took place in, allowed a secondary plot to be made (Figure 29). This plot was made by plotting k_{obs} versus [$^{-}$OH]. The fit of the points is linear, which reinforces that the hydrolysis *p*NPA is pseudo 2nd order in respect to OH^- with [Ca^{2+}] as the control. The slope on the Figure is the second order observed rate constant for the reaction in respect to [*p*NPA] and [$^{-}$OH] at the particular [Ca^{2+}]. The pseudo 2nd order rate constant in the presence of 333 mM $CaCl_2$ is 1130. $M^{-1}min^{-1}$ and the rate constant for 0 mM (Figure 29) is 638. $M^{-1}min^{-1}$. The second order observed rate constant increases as the amount of Ca^{2+} in solution

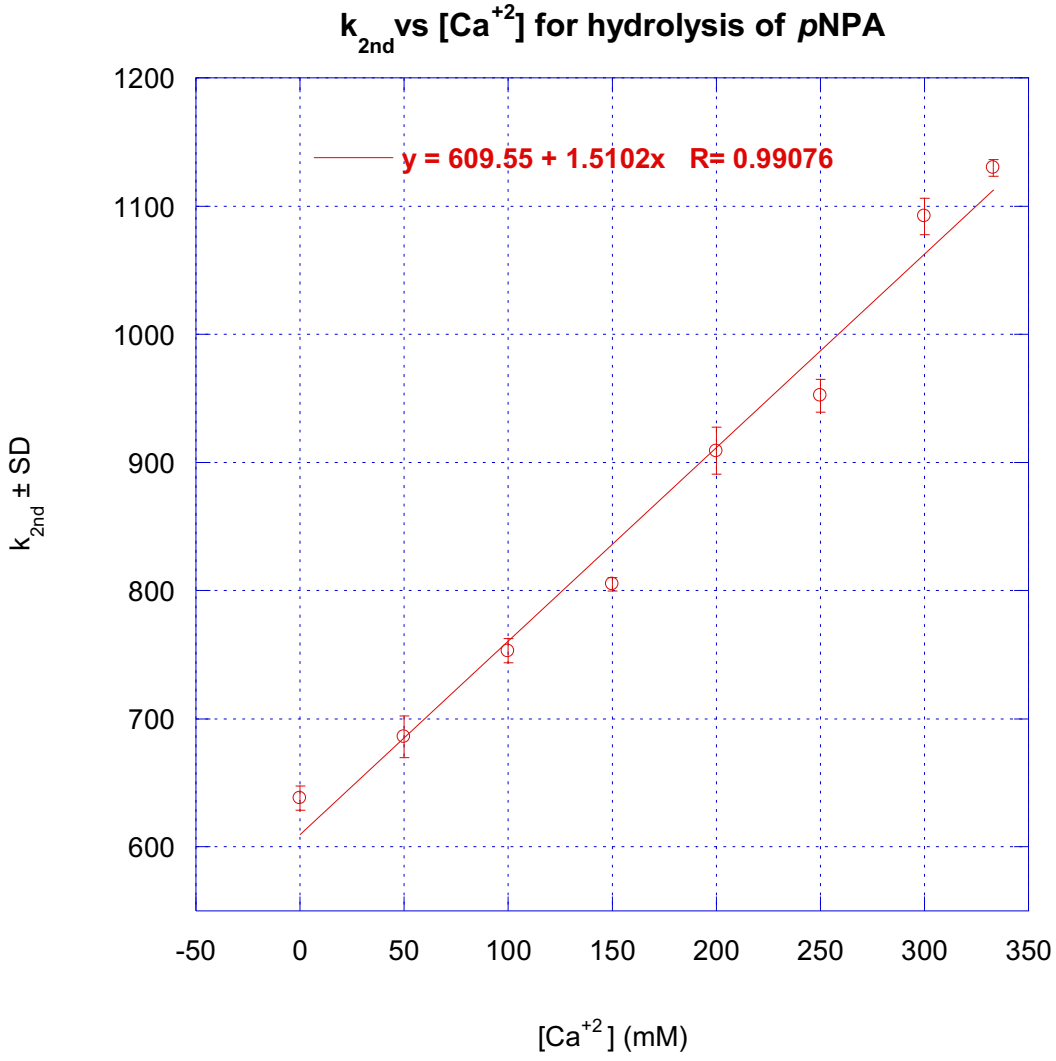
increases. The y-intercept is the rate the reaction takes place in the absence of ^{-}OH. The y-intercept is close to 0, which indicates that this reaction with H_2O is much slower than ^{-}OH.

The secondary plots displaying the relationship of the rate and [^{-}OH] for the varying concentrations of Ca^{2+} were used to make a tertiary plot comparing the k_{obs} with the [Ca^{2+}] (Figure 30). This plot was made by using the second order rate constants (slopes) from the k versus [^{-}OH] plots for and plotting it in respect to Ca^{2+} concentrations. This graph is used to assess changes in rates with changes in [Ca^{2+}] and is shown to follow a linear fit. The plot showed that the rate at which *p*NPA reacts in the absence of Ca^{2+} was found to be 638. $M^{-1}min^{-1}$, as indicated by the y-intercept. The rate at which 333 mM Ca^{2+} can catalyze the hydrolysis of *p*NPA was found to be 1130. $M^{-1}min^{-1}$. The linear fit also shows that there is one Ca^{2+} involved in the hydrolysis of *p*NPA. The rate at which [Ca^{2+}] increases *p*NPA hydrolysis was found by the slope and shown to be 1.51 M/M^2s^{-1}. Comparing 0 mM and 333 mM of [Ca^{2+}] shows that there is an increase in hydrolysis by approximately 77%.



Standard Deviations at varying [Ca^{2+}] concentrations			
[$^{-}$OH] Point	0 mM	150 mM	333 mM
1	0.0009	0.0018	0.0032
2	0.0137	0.0114	0.0247
3	0.0056	0.0103	0.0179
4	0.0279	0.0093	0.0248
5	0.0015	0.0049	0.0305

Figure 29 k versus [$^{-}$OH] for hydrolysis of *p*NPA at varying [$CaCl_2$]. (I'll make this in kaleidagraph when I get back to school) The [$^{-}$OH] were found by using the pHs of each of the solutions. The rates were found by using UV-vis spectrophotometry to measure the appearance of the product, *p*-nitrophenolate. All of the reactions contained 50 mM of CAPs buffer, 0.05 mM *p*NPA, and varying $CaCl_2$ and NaCl (to keep ionic strength to 1) at the different pHs. The slopes are the second order rate constants and it shows that the rate of hydrolysis increases with hydroxide concentration. As the Ca^{2+} concentration increases, the rate of hydrolysis also increases. There is a 77% increase in hydrolysis going from 0 mM $CaCl_2$ to 333 mM $CaCl_2$. The standard deviations are shown in the table, and the black arced lines are to clarify which point corresponds to what standard deviation. Each arc has the [$^{-}$OH] point labeled.



Standard Deviations at different [Ca^{2+}]								
[Ca^{2+}]	0	50	100	150	200	250	300	333
StDev	9.4	16.1	9.4	5.3	18.4	12.9	14.0	6.5

Figure 30 k_{obs} versus [Ca^{2+}] for hydrolysis of *p*NPA. The values on this plot were the second order rate constants for *p*NPA at different $CaCl_2$ concentrations. All of the reactions contained 50 mM CAPs buffer at varying pHs, 0.05 mM *p*NPA, varying $CaCl_2$ concentrations, and varying NaCl concentrations to keep ionic strength equal to 1. This plot shows that the rate of hydrolysis increases as the Ca^{2+} concentration increases. The slope is equal to the third order rate constant. The standard deviations are shown in the table below the figure. The [Ca^{2+}] values are lined up below the point they correspond to.

3.2 p-Nitroacetanilide Kinetics

The hydrolysis of *p*-nitroacetanilide was investigated at pH 11.00 without [Ca^{2+}] and with 333 mM [Ca^{2+}] (Figure 31). The hydrolysis rates were measured using UV-vis spectrophotometry to measure the rate at which the product *p*-nitroanilide appeared by using the increase in absorbance at 410 nm. Even though the maximum absorbance of *p*-nitroaniline is at 380 nm, that is interfered by the anilide's max absorbance at around 315 nm[37]. These reactions ran for 1700 minutes but due to the hydrolysis reaction occurring so slowly, that was not long enough for 100% product formation, and therefore not multiple half-lives. However, to investigate if there is an increase in hydrolysis rates, the initial rates were recorded using a linear fit and the *p*NAA concentration staying constant at 0.10 mM. What was found that the hydrolysis rates increased by approximately 164% going from 0 mM of [Ca^{2+}] to 333 mM of [Ca^{2+}] when adjusted for pH using mock solutions. The pH was adjusted by taking the rates of these reactions and dividing by the [$^{-}$OH] and comparing the two values. However, due to $CaCl_2$ decreasing the pH and these values are not adjusted for pH, the increase in hydrolysis may be higher due to the 333 mM [Ca^{2+}] sample may be at a lower pH than the 0 mM [Ca^{2+}] sample. The reason that the values started at 500 minutes rather than 0 because there needed to be time at the beginning of the trials for the conditions to stabilize.

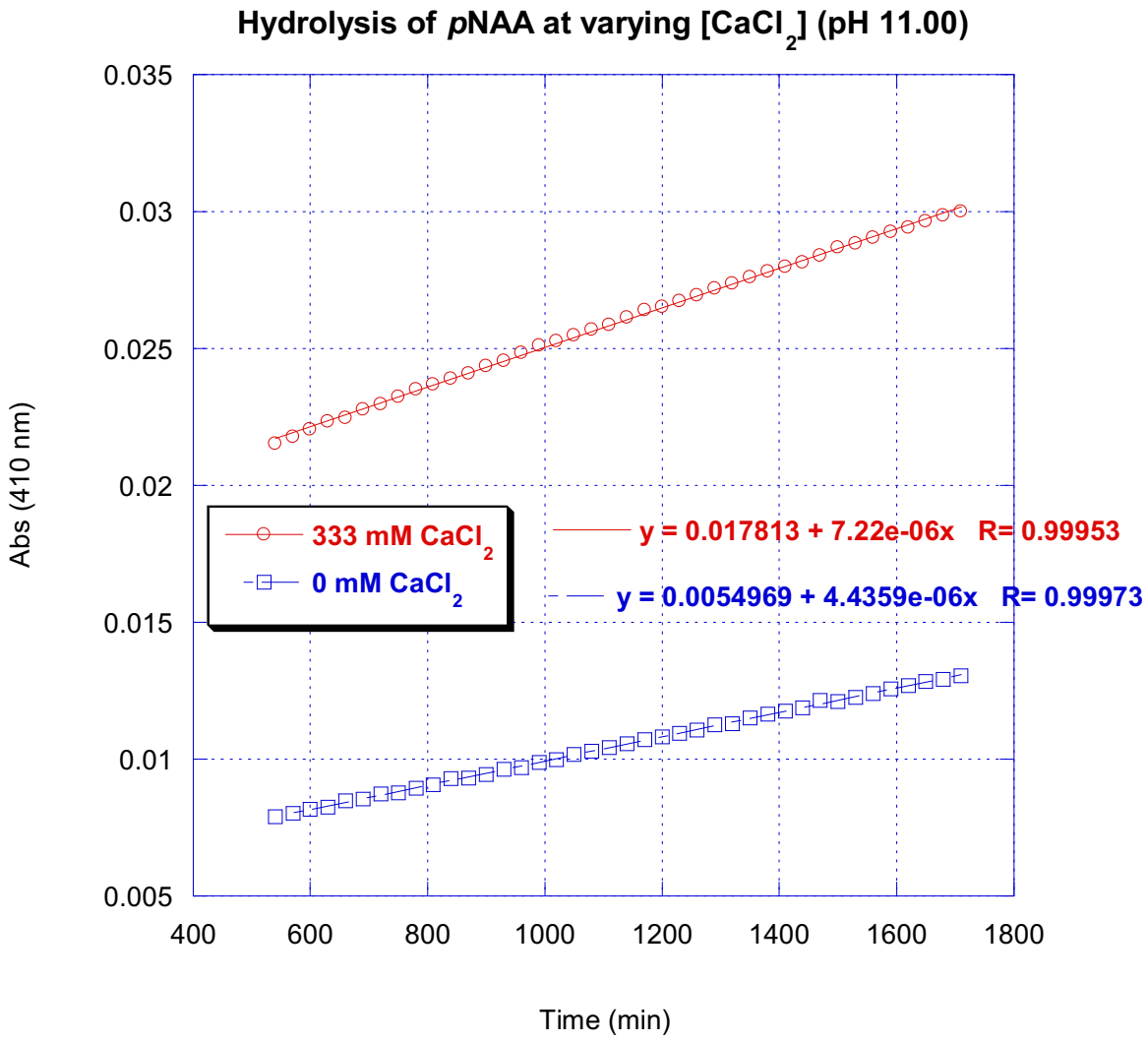


Figure 31 Hydrolysis of *p*NAA at pH 11 with and without Ca^{2+}. The hydrolysis of *p*NAA, was observed by measuring the appearance of the product *p*-nitroaniline at 410 nm using UV-Vis Spectrophotometry. All of the reactions contained 50 mM CAPs buffer at varying pH 11.00, 0.10 mM *p*NAA, varying $CaCl_2$ concentrations, and varying NaCl concentrations to keep ionic strength equal to 1. This plot shows that the rate of hydrolysis increases as the Ca^{2+} concentration increases. The slope is equal to the third order rate constant. There is a 63% increase in hydrolysis going from 0 mM $CaCl_2$ to 333 mM $CaCl_2$.

4.0 Discussion

4.1 p-Nitrophenyl Acetate

The molecule, *p*-nitrophenyl acetate (*p*NPA) was tested for metal ion catalysis with Ca^{2+} ions. This molecule does not exhibit a negative charge, but does have a partially negative oxygen at the carbonyl in the acetyl group that can potentially interact with Ca^{2+} (Figure 16). Another hypothesized way that Ca^{2+} can catalyze the hydrolysis of *p*NPA is by activating a hydroxide ion to make it a better nucleophile for the attack on the carbonyl carbon (Figure 16). As the pH increases, the rate of the hydrolysis reaction also increases, which indicates that hydroxide ions play a role in the hydrolysis of *p*NPA. This rate increase is expected because hydroxide ions are responsible for the nucleophilic attack on the carbonyl.

The log of rate vs pH graph was used to see how hydroxide is involved in the hydrolysis of *p*NPA (Figure 28). This graph had a linear fit plotted to it, to show the slope of the line formed from the data points. The slope of the line ends up indicating the number of hydroxide ions involved in the reaction because of the Henderson-Hasselbalch equation. The slope that we calculated was 0.98 with an R^2 of 0.99522. The slope is essentially equal to 1, so therefore one hydroxide ion is involved in this hydrolysis reaction. This agrees with the mechanistic action found by several different literature studies[25, 26, 31]. These literature sources all studied the mechanism of the hydrolysis of *p*NPA. What they found was that one hydroxide ion is being used in the hydrolysis of *p*NPA.

The secondary plot showing the rate versus [$^-$OH] was used to see how the rate of reaction compared to the actual concentration of $^-$OH rather than pH (Figure 29). The fact that the points were linear with low error confirms that the interaction between *p*NPA and OH^- is that of a first order reaction, both with and without Ca^{2+}. The slope of this graph indicates the second order

observed rate constant at the varying $CaCl_2$ concentrations, therefore the rate constant at a $CaCl_2$ concentration of 333 mM was 1131. $M^{-1}min^{-1}$ and 638. $M^{-1}min^{-1}$ at 0 mM of $CaCl_2$. The small increase indicates that Ca^{2+} is causing some type of catalysis in this system, but not by a dramatic amount. The y-intercept in this Figure shows the rate at which the hydrolysis of *p*NPA occurs in the absence of [$^{-}$OH]. This value is basically at 0 which means that attack by a water molecule is much slower than attack by hydroxide.

The tertiary plot, k_{obs} versus [Ca^{2+}] for the hydrolysis of *p*NPA, was used to see how much and how many Ca^{2+} ions catalyzed the hydrolysis of *p*NPA to *p*-nitrophenolate (Figure 30). This plot used a linear fit to quantitatively show how many Ca^{2+} ions interact with *p*NPA. It can be concluded that the binding to *p*NPA is much weaker than in phosphodiester systems, most likely due to the negative charge on phosphodiester bonds. At first glance it can be seen that the plot is shaped linearly. A straight line indicates a reaction with Ca^{2+} ion in the mechanism, if there were two involved in the mechanism, the plot would be parabolic. This graph is not parabolic so it can be concluded that there is only one Ca^{2+} ion involved in this catalysis.

This results contrasts with studies involving RNA and DNA models. These previous results have shown rectangular hyperbola indicating that there is one Ca^{2+} ion involved in the hydrolysis of molecules like RNA and UpG with intramolecular attack[20]. The reason that those models show a hyperbolic curve rather than a straight curve, even though they both show one Ca^{2+} ion is involved, is because the hyperbolic curve is showing signs of saturation. The reason that there are signs of saturation for that RNA molecule is because the Ca^{2+} ion concentration outnumbers the amount or RNA molecules significantly. As the concentration of Ca^{2+} increases there is less and less RNA molecules available to interact with, therefore showing signs of saturation. In the *p*NPA model, there is no signs of saturation occurring. The more likely possibility is due to there being

no interaction between the Ca^{2+} ion and the carbonyl oxygen, the catalysis is due to an interaction between Ca^{2+} and a hydroxide ion. Another possibility could be that the interaction between the carbonyl oxygen and a Ca^{2+} ion is so weak, that the Ca^{2+} ion concentration is just far too low to show any signs of saturation. For molecules requiring intermolecular mechanisms, a parabolic curve is shown, like with DNA and cAMP models[3]. This curve indicates that there are two Ca^{2+} ions involved in the mechanism. Even though *p*NPA hydrolysis occurs by an intermolecular mechanism, it was observed that only one Ca^{2+} ion was involved which is different to DNA models. The reason for this could be because in the DNA models, the Ca^{2+} is interacting with both the phosphodiester oxygens and the nucleophile, hydroxide. With *p*NPA, the Ca^{2+} is most likely interacting with only the hydroxide nucleophile and there is likely little to no interaction with the carbonyl oxygen.

However, just because we know that there is one Ca^{2+} ion involved in catalyzing this reaction, we do not know how exactly this Ca^{2+} is interacting in the mechanism. The Ca^{2+} could be interacting with the partially negative oxygen in the carbonyl in *p*NPA, as seen in the mammalian enzyme, serum paraoxonase (PON1)[14]. This enzyme was found to be able to catalyze both organophosphates and esters with the help of Ca^{2+} ion in the active site. The way in which this catalysis occurred between the two were also proposed to be very similar. The proposed mechanism was the Ca^{2+} ion interacting with the non-bridging oxygen in the organophosphates and the carbonyl oxygen in the ester. This leads to polarizing the attack site in both molecules and making it more likely for a nucleophilic attack. For this reason, it is plausible that in our system, a calcium ion can be interacting with the partially negative carbonyl on *p*NPA.

The interactions between amino acid esters and divalent metal nitrilotriacetate complexes were examined. They stated that it was widely known that metal ions play a role in amino acid

ester hydrolysis[39], due to either induction via the amino group by the metal ion, or that the metal ion interacts with the carbonyl oxygen. They used formation constants between different metal complexes and either glycine or ethyl valinate to determine the interaction between the metal complexes and the esters (Table 2). These values were calculated by using the titration curves of the different metal complexes with the molecules. Through their studies, they found that ethyl valinate's ester oxygen chelates to the metals, Mn and Pb, when they were present in the nitrilotriacetate complex. This is promising to our study because even though we are not working with a metal complex, there could still potentially be an interaction with calcium ion and the carbonyl oxygen in *p*NPA. Interestingly, when Cu or Zn was present in the nitrilotriacetate complex, it was found that the metals were coordinating with a hydroxide ion as well as the ester's carbonyl oxygen, which is another possibility on how calcium ions could interact with *p*NPA. Our results showed that calcium ions are present in the reaction and catalyze hydrolysis of *p*NPA. However, due to the low levels of catalysis, the calcium ions are believed to not interact with the carbonyl at all.

Table 2 Formation constants $[M(NTA)]^-$ with glycine and ethyl valinate[39].

M^{2+}	Log K_{OH}[a]	Log K_{fEtVal}[b]	Log K_{fGly}[a]	R
Mn		2.39 ± 0.02	2.24 ± 0.005	0.7
Co		1.88 ± 0.014	3.65 ± 0.014	59
Ni		2.03 ± 0.011	4.95 ± 0.011	830
Cu	4.39 ± 0.01	2.88 ± 0.002	5.46 ± 0.008	360
Zn	3.55 ± 0.05	1.58 ± 0.08	3.64 ± 0.007	110
Pb		1.55 ± 0.10	1.93 ± 0.009	2.4

[a]I=0.073 M, [b]I=0.077 M, R=K_{fGly}/K_{fEtVal}, M^{2+}=0.0067 M, glycine or ethyl valinate=0.0067 M.

Calcium ions could also be involved in the hydrolysis mechanism by activating a hydroxide ion, to make it more nucleophilic for the attack on the carbonyl. A research group in China studied the catalytic hydrolysis of *p*NPA and *p*-nitrophenyl picolinate (*p*NPP) (Figure 17) using Cu(II) and Zn(II) complexes in a Schiff base ligand (Figure 19)[28]. They found that with *p*NPA, the Cu(II)

complex has a very little effect on increasing catalysis. However, the Cu(II) complex increases the catalysis of *p*NPP by approximately 80 fold. The reason why the Cu(II) complex increased the hydrolysis rate of *p*NPP much more than *p*NPA is because of the picolinate group on the *p*NPP. *p*NPA only contains a carbonyl which has a low coordinative ability. *p*NPP however has both a carbonyl and a picolinate group, which has a very strong coordination ability. The nitrogen was able to coordinate with the Cu(II) complex, which polarizes the carbonyl, making it a better electrophile. The very little coordination ability with *p*NPA is why there is little catalysis done by the Cu(II) complex. This indicates that in our reaction that the Ca^{2+} may not interact with carbonyl in the *p*NPA at all because of the low coordination ability of the carbonyl. This is consistent with the low levels of catalysis and lack of saturation behavior, in our model system with Ca^{2+} and *p*NPA.

The Cu(II) complex did not show a catalytic effect for the hydrolysis of *p*NPA, but the Zn(II) complex did[28]. The Zn(II) complex increased the hydrolysis of *p*NPA by approximately 9 fold and approximately 50 fold for *p*NPP. The biggest difference between the Cu(II) and Zn(II) complex is that the Zn(II) complex coordinates with a water molecule, which can lead to different mechanisms of catalysis. They found that when the Zn(II) water complex was present in solution, the active species was the metal bound hydroxide rather than the hydroxide ions found in solution. One possibility why *p*NPP is catalyzed more than *p*NPA by the Zn(II) complex could be due to the picolinate group being electron withdrawing, and the methyl group being electron donating. An electron withdrawing group would stabilize the negative charge on the transition state more than an electron donating group, so therefore *p*NPP would be catalyzed more. It is believed that *p*NPP gets hydrolyzed via an intramolecular Zn(II) activated hydroxide attack due to the Zn(II)-OH^- complex interacting with the nitrogen on the picolinate group. *p*NPA however gets hydrolyzed

via an intermolecular Zn(II) activated hydroxide attack because the complex cannot interact with *p*NPA. In my experiment, the Ca^{2+} ions could be only interacting with free hydroxide ions in the basic solutions and therefore be done by an intermolecular attack. Ca^{2+} would also be a worse catalyst than Zn^{2+} because Ca^{2+} is a weaker Lewis acid and therefore a weaker ^{-}OH activator[40]. There could be no interaction between Ca^{2+} ions and *p*NPA, like this paper suggests with metal complexes. So, the increase in hydrolysis rates in our reaction could be due to Ca^{2+} ions activating the nucleophilic hydroxide, rather than interacting with *p*NPA.

The same group of scientists conducted another study that was very similar to the Zn(II) and Cu(II), in Schiff base ligand complexes[29]. This study still uses Zn(II) and Cu(II) complexes, except this time, the reactions were also done in CTAB micellar solutions (Figure 18). Using a micellar solution to make a metallomicelle is advantageous because it is able to simulate the conditions in which reactions take place in metalloenzymes. Their findings were very similar to what they found earlier, *p*NPP has a large increase in catalysis due to the Cu(II) and Zn(II) complexes, having a 3580-fold and 6847-fold increase in rate, respectively, and *p*NPA has a low increase in catalysis due to the complexes, having a 22-fold and 54-fold increase in rate, respectively. The reason for this was the same, it was because *p*NPA is known to have a low ability to coordinate with a metal ion. For this reason, the hydrolysis reaction for *p*NPP happens with an intramolecular hydroxide ion in the metal complex, and *p*NPA hydrolysis occurs via an intermolecular hydroxide ion. Intramolecular reactions are much faster than intermolecular reactions.

The last possibility is that a Ca^{2+} ion can do both, interacting with the carbonyl oxygen and bringing in an activated hydroxide to be used for a nucleophilic attack. As stated earlier, when Cu or Zn was present in a nitrilotriacetate complex, the metals chelated to both the ester's oxygen as

well as a hydroxide[39]. Also stated earlier, with molecules with higher abilities to coordinate with a metal ion, like with *p*NPP, the hydrolysis reaction gets catalyzed more because the reaction is now intramolecular rather than intermolecular, due to the metal coordinating with both the substrate and a hydroxide ion[28, 29]. There is a possibility that when free in solution that Ca^{2+} may be able to coordinate with *p*NPA, even though it was believed not possible in metal complexes. This catalyzes the reaction because not only does the carbonyl carbon becomes more electrophilic due to induction from the metal ions, but also the hydroxide ion becomes more nucleophilic for the attack.

The maximum rate with 333 mM of Ca^{2+} was found to be 1131. $M^{-1}min^{-1}$ and compared to the rate at 0 mM of Ca^{2+}, which is 638. $M^{-1}min^{-1}$, shows that Ca^{2+} increases the rate of hydrolysis of *p*NPA by about 77% or 1.77-fold. The third order rate constant is the slope of this plot and was found to be 1.51 M/M^2s^{-1}. These values are not a lot when you compare it to other types of catalyses and especially when compared to enzymes. Past work in Dr. Cassano's lab showed that Ca^{2+} ions can catalyze the rate of hydrolysis for phosphodiesters by up to 900 fold[22]. This 900 fold however was due to interaction between two Calcium ions. With *p*NPA, it was found through the pH vs log(k) plot that only one Ca^{2+} ion is involved in the hydrolysis of *p*NPA. However, even the first Ca^{2+} catalyzes the phosphodiester by 90 fold which is a lot greater than the 1.77 fold increase with *p*NPA. A reason for why phosphodiesters have an increase in catalysis by Ca^{2+} ions could be due to the fact that phosphodiesters contain a negative charge[3, 19]. Also with phosphodiesters, there is an additional build up on the non-bridging oxygen in the transition state and the phosphorene intermediate. Ca^{2+} ions would be drawn to the negative charge and will help stabilize the growing negative charge on the intermediate and transition states, therefore leading to a greater catalysis. This negative charge could interact with the Ca^{2+} ions, and this calcium ion could also coordinate

with a hydroxide ion to have an intramolecular hydroxide attack, to hydrolyze the reaction. This type of behavior in metal ion catalysis agrees with what is found in phosphodiesterases[11]. Metal ions are commonly found in phosphodiesterase active sites, to aid in the hydrolysis of phosphodiester bonds.

Going back to *p*NPA, other systems to look at for catalysis of metal ions are metal ion complexes. Like stated before with research on Zn(II) and Cu(II) complexes, these complexes are able to increase hydrolysis rates for *p*NPA and *p*NPP[28, 29]. They found that *p*NPP had a larger increase in hydrolysis rates due to the nitrogen in the picolinate group being able to interact with the complexes. The increases in catalysis with the metal complexes and with my system of free Ca^{2+} ions in solution will be compared. With the Zn(II) ion in the Schiff base ligand, forming a ZnL-H_2O complex, hydrolysis rates increased by approximately 9 fold[28] and the rate was increased by approximately 50 fold with the Zn(II) complex in a CTAB micellar solution[29]. Both of these conditions are higher than the 1.77 fold increase found in my studies (Table 3). This could be due to the complexes activating the hydroxide ions better than Ca^{2+} ions do. Another possibility could be that different metal ions have greater catalytic abilities than others[40]. For example, it was found that with the hydrolysis of a *p*-nitrophenyl phosphate derivative, different metal ions had different amounts of catalysis (Figure 32). Ca^{2+} was the weakest catalyst that they studied having only a 3-fold increase in the hydrolysis rate of the *p*-nitrophenyl phosphate derivative. Mg^{2+} and Zn^{2+} had a significantly higher fold catalysis with 46-fold and 150-fold respectively. The study also examined the metal ions, Pb^{2+}, Eu^{2+}, Tb^{2+}, and Yb^{2+}, in conjunction with different ligands. What they found with them is that the ligands always increased catalysis even further for the hydrolysis of the *p*-nitrophenyl phosphate derivative. It is also important to note that this substrate has a negative charge which is believed to bind to the metal ions which is how catalysis occurs with

metal ions. The micellar solutions were expected to catalyze the reaction more than complexes and ions free in solution[29]. The reason is because micelles simulate the insides of an enzyme, for the fact that everything is closer together and makes the reaction more likely to occur.

Table 3 The increase in hydrolysis rates of *p*NPA with Ca^{2+} versus other model systems.

Model System	Fold-Catalysis
Ca^{2+}	1.77
ZnL – H_2O complex	9 [28]
Cu(II) complex in CTAB	22 [29]
Zn(II) complex in CTAB	50 [29]

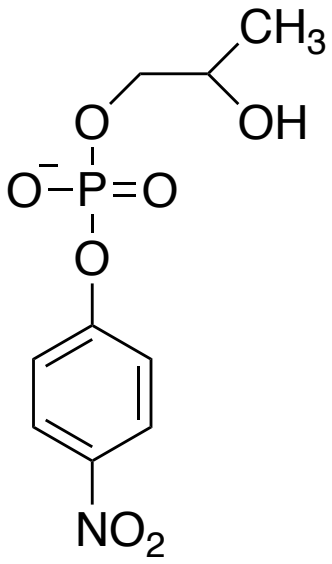


Figure 32 *p*-nitrophenyl phosphate derivative that was tested with a variety of metal ions[40]. This substrate was tested with a variety of metal ions to see which ones catalyzed the hydrolysis of the substrate more effectively. It was found that Ca^{2+} was the weakest catalyst and Mg^{2+} and Zn^{2+} were significantly better. It is believed that the metal ions can bind to the negative charge on the non-bridging oxygen, which leads to catalyzing the hydrolysis reaction.

The amount that Ca^{2+} catalyzes *p*NPA hydrolysis is very low when compared to other systems like with the RNA model, UpG[20], and the DNA model, cAMP[3] (Table 4). The molecule *p*NPA gets hydrolyzed by 1.77 fold when [Ca^{2+}] is increased from 0 to 333 mM. UpG however hydrolysis increases by 326 fold and cAMP hydrolysis increases by 820 fold from the same increase of Ca^{2+} concentration. This is most likely do to the fact that both the RNA and DNA

models showed interaction between the Ca^{2+} ion and the molecule being hydrolyzed, which leads to a much greater increase in catalysis. The reason that cAMP has a higher catalysis than UpG is probably due to the fact that cAMP hydrolysis occurs via a two Ca^{2+} ion mechanism and UpG only utilizes one Ca^{2+} ion.

Table 4 The hydrolysis of different molecules using $CaCl_2$

Molecule	Fold-Catalysis
*p*NPA	1.77
UpG[20]	326[20]
cAMP[3]	820[3]

All of these model systems had μ=1 and had [Ca^{2+}] ranging from 0 mM to 333 mM

So far, the major conclusions of this data is that Ca^{2+} ions do catalyze the hydrolysis of *p*NPA via a third order reaction, where the concentration of *p*NPA, hydroxide, and Ca^{2+} ions will determine the rate. The increase in hydrolysis was found to be 77% when going from 0 mM to 333 mM $CaCl_2$. Also, one Ca^{2+} ion was involved in the mechanistic action of this catalysis, but it is still unknown in exactly how this metal interacts with the substrate. The reason that Ca^{2+} ions had such a low effect on hydrolysis rates for *p*NPA compared with phosphodiesters could be because *p*NPA lacks a negative charge, where phosphodiester's non-bridging oxygens contain a negative charge. Ca^{2+} ions can interact with the negative charges on the oxygens and bring in a hydroxide to attack the phosphodiester to cleave it. Another reason could be due to the fact that *p*NPA's hydrolysis product, *p*-nitrophenol, has great leaving group ability and phosphodiesters have a very poor leaving group. Due to the poor leaving group on phosphodiesters, metal ions are needed more for hydrolysis to occur and in *p*NPA, hydrolysis reactions can occur without metal ions present.

4.2 p-Nitroacetanilide

Further work to investigate the more specifics for how Ca^{2+} ions actually catalyze the hydrolysis of *p*NPA should be done. A way to study this is by working with the molecule *p*-nitroacetanilide (*p*NAA). The structure is very closely related to *p*NAA, the only difference is that

instead of being an ester, it has a nitrogen, making it an amide. Past research has showed that where *p*NPA hydrolysis is a concerted reaction, *p*NAA hydrolysis is a stepwise reaction[31]. This was done by measuring the binding energies of hydroxide and the molecule. They found that *p*NPA has a single transition state, showing a concerted reaction, and *p*NAA was found to have two transition states, which indicates a stepwise reaction. This is interesting to look at because one reason we think that Ca^{2+} has such a low effect on the hydrolysis rates of *p*NPA, especially compared to phosphodiesters is because of how good of a leaving group that *p*NPA has. The leaving groups of phosphodiesters are very poor, so they need the Ca^{2+} ion in order to even get the reaction to occur, this is not the case for *p*NPA, as the reaction can easily occur without Ca^{2+} present. *p*NAA is different though because it is stepwise, indicating that the leaving group, 4-nitroaniline is not as good compared to *p*-nitrophenolate. This could mean that Ca^{2+} ions could potentially have a larger effect for *p*NAA hydrolysis because the Ca^{2+} could stabilize the negative charged tetrahedral intermediate. Also, because the reaction is stepwise, the intermediate contains a negative charge on the carbonyl oxygen. However, there is still not a huge increase in catalysis with Ca^{2+}. If this is the case, then it is more plausible that Ca^{2+} ions are only able to catalyze the reaction via activating a hydroxide ion and does not interact with the carbonyl oxygen whatsoever.

The effect of Ca^{2+} ions on the hydrolysis of the anilide, *p*-nitroacetanilide was investigated (Figure 31). These results are only preliminary, so therefore will determine if it is worth looking into or not. Only the initial rates for the hydrolysis of *p*NAA were found by measuring the slopes of the UV-Vis Spectrophotometry studies. The results show that there could be a 164% or 2.64 fold increase in hydrolysis of *p*NAA when 0 mM [Ca^{2+}] is used compared to 333 mM of [Ca^{2+}]. Although this 2.64 fold increase in catalysis is higher than the 1.77 found with *p*NPA, it still shows that there is likely not much interaction between the carbonyl oxygen and the Ca^{2+} ion. If there

was an interaction in this reaction, the fold increase would be substantially higher due to *p*NAA having a negatively charged intermediate and *p*NPA having no intermediate at all. The increased catalysis could have been occurred because of the reaction happening more slowly and there being an intermediate present. However, these findings are only preliminary, in order to see the true effect of [Ca^{2+}] more trials need to be ran, similarly to the *p*NPA studies. This would allow the hydrolysis rates to be found in respect to [$^{-}$OH] as well as in respect to [Ca^{2+}]. Future directions include doing more trials with *p*NAA at more calcium concentrations and different pHs. This would allow for rate constants to be found for *p*NAA in respect to [$^{-}$OH] and [Ca^{2+}] in order to see the actual effect of [Ca^{2+}] on the hydrolysis of *p*NAA. This would allow the rates to be able to compare better with both literature and the values found for *p*NPA.

5.0 References

1. Kraut, J., How do enzymes work? *Science* **1988,** *242* (4878), 533-540.
2. Hammes, G. G., How do enzymes really work? *Journal of Biological Chemistry* **2008,** *283* (33), 22337-22346.
3. Alex, J., Ca 2+ Catalyzed Adenosine 3', 5'-Cyclic Monophosphate Hydrolysis: Insights into the Role of Metals in Phosphodiesterase Active Sites. In *A Thesis in Biochemistry and Molecular Biology*, Cassano, A., Ed. Drew University: Madison, NJ, 2018; pp 1-71.
4. McCall, K. A.; Huang, C.-c.; Fierke, C. A., Function and mechanism of zinc metalloenzymes. *The Journal of nutrition* **2000,** *130* (5), 1437S-1446S.
5. Lassila, J. K.; Zalatan, J. G.; Herschlag, D., Biological phosphoryl-transfer reactions: understanding mechanism and catalysis. *Annual review of biochemistry* **2011,** *80*, 669-702.
6. Mikkola, S.; Lönnberg, T.; Lönnberg, H., Phosphodiester models for cleavage of nucleic acids. *Beilstein journal of organic chemistry* **2018,** *14* (1), 803-837.
7. Huang, J.-C.; Svoboda, D. L.; Reardon, J. T.; Sancar, A., Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5'and the 6th phosphodiester bond 3'to the photodimer. *Proceedings of the National Academy of Sciences* **1992,** *89* (8), 3664-3668.
8. Fothergill, M.; Goodman, M. F.; Petruska, J.; Warshel, A., Structure-energy analysis of the role of metal ions in phosphodiester bond hydrolysis by DNA polymerase I. *Journal of the American Chemical Society* **1995,** *117* (47), 11619-11627.

9. Groll, D. H.; Jeltsch, A.; Selent, U.; Pingoud, A., Does the restriction endonuclease Eco RV employ a two-metal-ion mechanism for DNA cleavage? *Biochemistry* **1997,** *36* (38), 11389-11401.
10. Imhof, P.; Fischer, S.; Smith, J. C., Catalytic mechanism of DNA backbone cleavage by the restriction enzyme EcoRV: a quantum mechanical/molecular mechanical analysis. *Biochemistry* **2009,** *48* (38), 9061-9075.
11. Lönnberg, H., Cleavage of RNA phosphodiester bonds by small molecular entities: a mechanistic insight. *Organic & biomolecular chemistry* **2011,** *9* (6), 1687-1703.
12. Hernick, M.; Fierke, C. A., Zinc hydrolases: the mechanisms of zinc-dependent deacetylases. *Archives of biochemistry and biophysics* **2005,** *433* (1), 71-84.
13. Folk, J.; Cole, P.; Mullooly, J., Mechanism of action of guinea pig liver transglutaminase III. The metal-dependent hydrolysis of p-nitrophenyl acetate; further observations on the role of metal in enzyme activation. *Journal of Biological Chemistry* **1967,** *242* (11), 2615-2621.
14. Harel, M.; Aharoni, A.; Gaidukov, L.; Brumshtein, B.; Khersonsky, O.; Meged, R.; Dvir, H.; Ravelli, R. B.; McCarthy, A.; Toker, L., Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nature Structural and Molecular Biology* **2004,** *11* (5), 412.
15. Lehninger, A., Role of metal ions in enzyme systems. *Physiological reviews* **1950,** *30* (3), 393-429.
16. Kurz, J. L., Transition state characterization for catalyzed reactions. *Journal of the American Chemical Society* **1963,** *85* (7), 987-991.
17. Klein, D., Aldol Reactions. In *Organic Chemistry*, Second ed.; John Wiley and Sons, Inc.: Johns Hopkins University, 2015; p 1057.
18. Suh, J., Model studies of metalloenzymes involving metal ions as Lewis acid catalysts. *Accounts of Chemical Research* **1992,** *25* (7), 273-279.
19. Gomez-Tagle, P.; Vargas-Zúñiga, I.; Taran, O.; Yatsimirsky, A. K., Solvent effects and alkali metal ion catalysis in phosphodiester hydrolysis. *The Journal of organic chemistry* **2006,** *71* (26), 9713-9722.
20. Messina, K. Investigation of the Mechanism of Ca^{2+} Catalyzed RNA Phosphodiester Hydrolysis. Drew University, Drew University, 2014.
21. Blasko, A.; Bruice, T. C., Recent studies of nucleophilic, general-acid, and metal ion catalysis of phosphate diester hydrolysis. *Accounts of chemical research* **1999,** *32* (6), 475-484.
22. Kirk, B. A.; Cusack, C. L.; Laager, E.; Rochlis, E.; Thomas, T.; Cassano, A. G., Mononuclear and dinuclear mechanisms for catalysis of phosphodiester cleavage by alkaline earth metal ions in aqueous solution. *Journal of inorganic biochemistry* **2010,** *104* (2), 207-210.
23. Hershfield, R.; Bender, M. L., Nucleophilic and metal ion acceleration of ester hydrolysis in a hydrophobic complex. Reactive enzyme model system. *Journal of the American Chemical Society* **1972,** *94* (4), 1376-1377.
24. Kroll, H., The Participation of Heavy Metal Ions in the Hydrolysis of Amino Acid Esters1. *Journal of the American Chemical Society* **1952,** *74* (8), 2036-2039.
25. Xie, D.; Zhou, Y.; Xu, D.; Guo, H., Solvent effect on concertedness of the transition state in the hydrolysis of p-nitrophenyl acetate. *Organic letters* **2005,** *7* (11), 2093-2095.

26. Hengge, A. C.; Hess, R. A., Concerted or stepwise mechanisms for acyl transfer reactions of p-nitrophenyl acetate? Transition state structures from isotope effects. *Journal of the American Chemical Society* **1994,** *116* (25), 11256-11263.
27. Perrin, D. D.; Dempsey, B.; Serjeant, E. P., *pKa prediction for organic acids and bases*. Springer: 1981; Vol. 1.
28. Kou, X.; Cheng, S.; Du, J.; Yu, X.; Zeng, X., Catalytic hydrolysis of carboxylic acid esters by Cu (II) and Zn (II) complexes containing a tetracoordinate macrocyclic Schiff base ligand in Brij35 micellar solution. *Journal of Molecular Catalysis A: Chemical* **2004,** *210* (1-2), 23-29.
29. Juan, D.; Bingying, J.; Xingming, K.; Xiancheng, Z.; Qingxiang, X., Enhanced hydrolysis of carboxylic acid esters catalyzed by metallomicelles made of Cu (II) and Zn (II) complexes. *Journal of colloid and interface science* **2002,** *256* (2), 428-434.
30. Stewart, R.; Dolman, D., A comparison of the acidity and basicity of aromatic amines. *Canadian Journal of Chemistry* **1967,** *45* (9), 925-928.
31. Chong, L. T.; Bandyopadhyay, P.; Scanlan, T. S.; Kuntz, I. D.; Kollman, P. A., Direct hydroxide attack is a plausible mechanism for amidase antibody 43C9. *Journal of computational chemistry* **2003,** *24* (12), 1371-1377.
32. Bender, M. L.; Pollack, R. M., Alkaline Hydrolysis of p-Nitroacetanilide and p-Formylacetanilide. *Journal of the American Chemical Society* **1970,** *92* (24), 7190-7194.
33. Stein, R. L., Enzymatic hydrolysis of p-nitroacetanilide: mechanistic studies of the aryl acylamidase from Pseudomonas fluorescens. *Biochemistry* **2002,** *41* (3), 991-1000.
34. Broxton, T. J.; Nasser, A., Micellar catalysis of organic reactions. Part 37. A comparison of the catalysis of ester and amide hydrolysis by copper-containing micelles. *Canadian journal of chemistry* **1997,** *75* (2), 202-206.
35. Sayre, L. M., Metal ion catalysis of amide hydrolysis. *Journal of the American Chemical Society* **1986,** *108* (7), 1632-1635.
36. Aldrich, S., Enzymatic Assay of α-GALACTOSIDASE
(EC 3.2.1.22). In *Product Information*, 8/22/97 ed.; Sigma Aldrich: Saint Louis, Missouri, 1997; p 3.
37. Aldrich, S., N-METHOXYSUCCINYL -ALA-ALA-PRO-VAL
P-NITROANILIDE. In *Product Information*, 3/12/99 ed.; Sigma Aldrich: Saint Louis, Missouri, 1999; p 2.
38. Moradi, N.; Ashrafi-Kooshk, M. R.; Ghobadi, S.; Shahlaei, M.; Khodarahmi, R., Spectroscopic study of drug-binding characteristics of unmodified and pNPA-based acetylated human serum albumin: does esterase activity affect microenvironment of drug binding sites on the protein? *Journal of Luminescence* **2015,** *160*, 351-361.
39. Hopgood, D.; Angelici, R. J., Equilibrium and stereochemical studies of the interactions of amino acids and their esters with divalent metal nitrilotriacetate complexes. *Journal of the American Chemical Society* **1968,** *90* (10), 2508-2513.
40. Breslow, R.; Huang, D.-L., Effects of metal ions, including Mg2+ and lanthanides, on the cleavage of ribonucleotides and RNA model compounds. *Proceedings of the National Academy of Sciences* **1991,** *88* (10), 4080-4083.