Investigation of the Iso-Orotate decarboxylation reaction mechanism: Enzymatic and non-enzymatic and study of the isotope effects involved in its mechanism

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Thesis Abstract

Some organisms, such as *Neurospora crassa*, have a unique capability of producing uracil through the thymine salvage pathway. The enzyme involved in the final step of this pathway, Iso-orotate decarboxylase, catalyzes conversion of Iso-orotate to uracil through a decarboxylation reaction. The project done in the lab mainly concentrated on kinetic studies on the non-enzymatic conversion of Iso-orotate to uracil. Apart from this isotope effects involved in the reaction have also been studied to get a comprehensive understanding on the mechanistic details involved in the decarboxylation reaction catalyzed by Iso-orotate decarboxylase. This work will provide a foundation for future mechanistic studies with the enzyme.

Table of Contents

Acknowledgements

First I would like to thank my parents who have always been a source of support and inspiration for me. I love you dad and mom. I would not have done this without you. I take this opportunity to thank my brother for all his love and confidence in me. I also would like to express my gratitude to my advisor Dr. Jeffrey A. Smiley for all his support and patience. Then last but not least I would like to show my undying gratitude to all my friends for their faith in me.

List of Tables

List of Figures

List of Symbols and Abbreviations

DNA .....Deoxyribo Nucleic Acid.

RNA .....Ribo Nucleic Acid

PRPP.....5-phosphoribosyl-1-pyrophosphate

IMP.....Inosine 5'monophosphate

ATP.....Adenosine Tri Phosphate

THF.....Tetrahydrofolate

GMP.....Guanosine Mono Phosphate

GTP.....GuanosineTriPhosphate

AMP..... Adenosine MonoPhosphate

CPS..... Carbomyl Phosphate Synthetase

UMP.....Uridylate MonoPhosphate

OMP.....OrotidylateMonoPhosphate

UTP.....UridylatetriPhosphate

UDP.....UridylateDiPhosphate

IOA.....Iso-Orotate

IDCase.....Iso-Orotate Decarboxylas

HPLC.....High Pressure Liquid Chromatography

UV..... Ultraviolet

IRMS.....Isotope Ratio Mass Spectroscopy

KIE.....Kinetic Isotopic Effects

nmol.....Nanomoles

δ .....Delta

μ .....micro

Chapter 1

Introduction

The large amount of information required to maintain the complex system of living organisms is stored in nucleic acids. A nucleic acid is a complex, high molecular-weight biochemical macromolecule composed of nucleotide chains that convey genetic information. The backbone of a nucleic acid is made of alternating sugar and phosphate groups bonded together in a long chain. Each of the sugar groups in the backbone is attached to a third type of molecule called a nucleotide base. Nucleic acids contain four nucleotide bases. The nucleotide bases serve as a sort of genetic alphabet on which the structure of each protein in our bodies is encoded. The nucleotide bases are of two types: purines and pyrimidines. The purines that are present are adenine and guanine and the pyrimidines that are present are thymine, cytosine and uracil.[1]

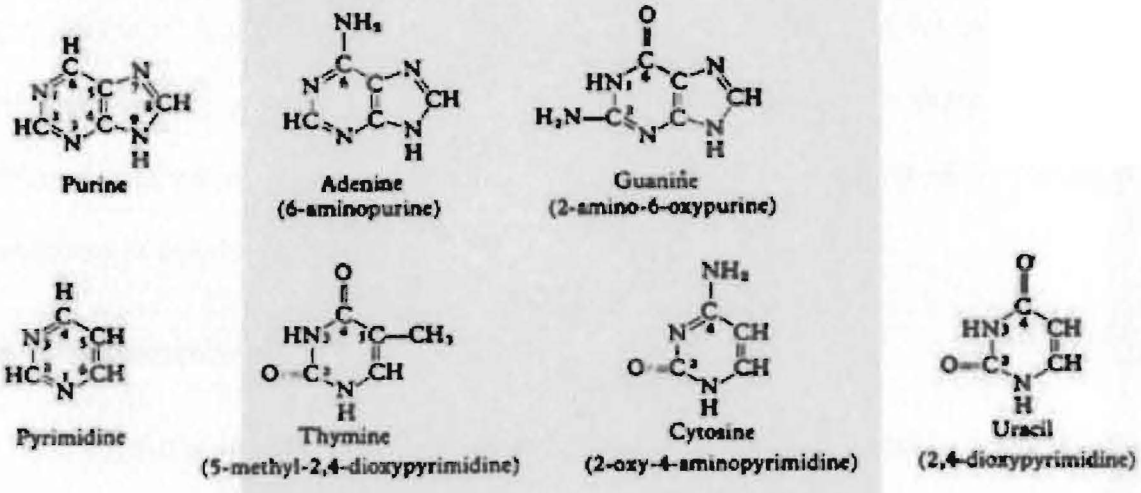


Figure 1-1: Purines and pyrimidines.

DNA

The genetic information is stored in the molecule deoxyribonucleic acid or DNA in most of the living organisms. DNA contains the sugar deoxyribose as its backbone. The four nucleotide bases that occur in DNA are adenine (A), cytosine (C), guanine (G), and thymine (T). The four nitrogenous bases of DNA are arranged along the sugar-phosphate backbone in a particular order (the DNA sequence), encoding all genetic instructions for an organism. Adenine (A) pairs with thymine (T), while cytosine (C) pairs with guanine (G). The two DNA strands are held together by hydrogen bonds between the bases.

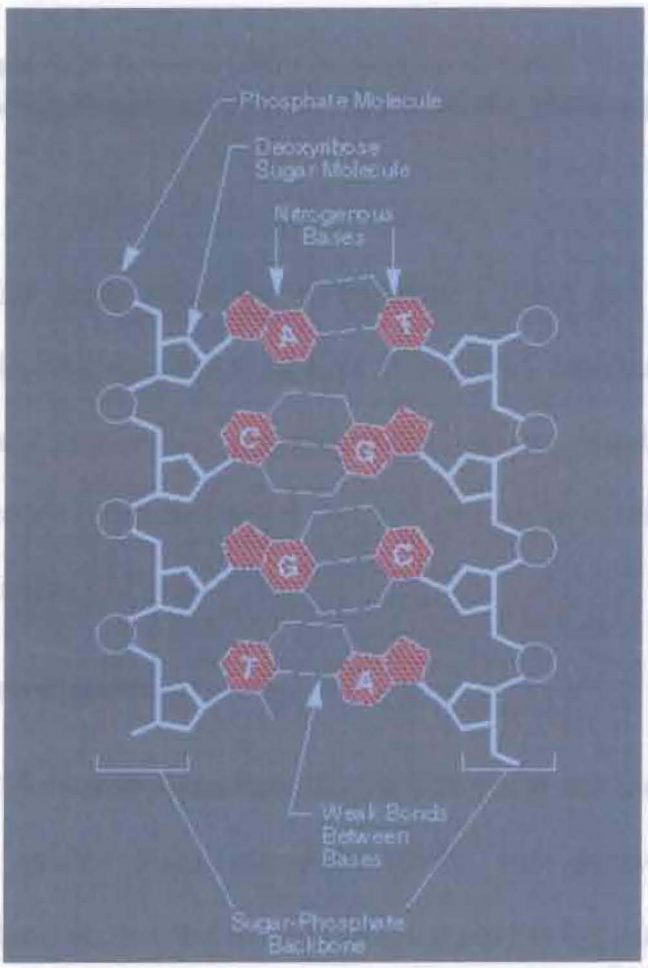


Figure 1-2: Structure of DNA. Source: http://www.visionlearning.com/library/module_viewer.php?mid=63

DNA is a double helix. The double stranded DNA has the unique ability that it can make exact copies of itself, or self-replicate.

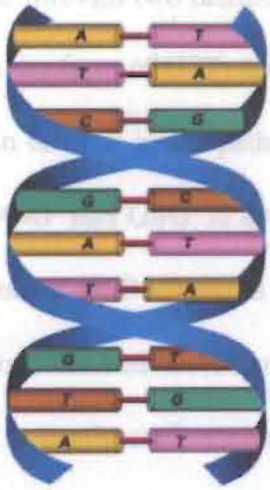


Figure 1-3: Double helix structure of DNA. Source: http://www.visionlearning.com/library/module_viewer.php?mid=63

RNA

RNA contains sugar ribose as its backbone. RNA has a sugar-phosphate backbone with nucleotides attached to it. Like DNA, RNA contains the bases adenine (A), cytosine (C), and guanine (G); however, RNA does not contain thymine, instead, RNA's fourth nucleotide is the base uracil (U). RNA's may be considered as vectors or translators of information.

***De novo* biosynthesis of purines:**

Synthesis of the purine nucleotides begins with PRPP and leads to the first fully formed nucleotide, inosine 5'-monophosphate (IMP). This pathway is diagrammed below. The purine base without the attached ribose moiety is hypoxanthine. The purine base is built upon the ribose by several amidotransferase and transformylation reactions. The synthesis of IMP requires five moles of ATP, two moles of glutamine, one mole of glycine, one mole of CO_2, one mole of aspartate and two moles of formate. The formyl

moieties are carried on tetrahydrofolate (THF) in the form of N^5, N^{10}-methenyl-THF and N^{10}-formyl-THF. IMP represents a branch point for purine biosynthesis, because it can be converted into either AMP or GMP through two distinct reaction pathways. The pathway leading to AMP requires energy in the form of GTP; that leading to GMP requires energy in the form of ATP. The utilization of GTP in the pathway to AMP synthesis allows the cell to control the proportions of AMP and GMP to near equivalence. The accumulation of excess GTP will lead to accelerated AMP synthesis from IMP instead, at the expense of GMP synthesis. Conversely, since the conversion of IMP to GMP requires ATP, the accumulation of excess ATP leads to accelerated synthesis of GMP over that of AMP.[2]

Formation of AMP and GMP from IMP

IMP is a common precursor to both AMP and GMP.

Formation of adenylate (AMP): The C-6 carbonyl group of inosinate is replaced with the amino group from Asp.

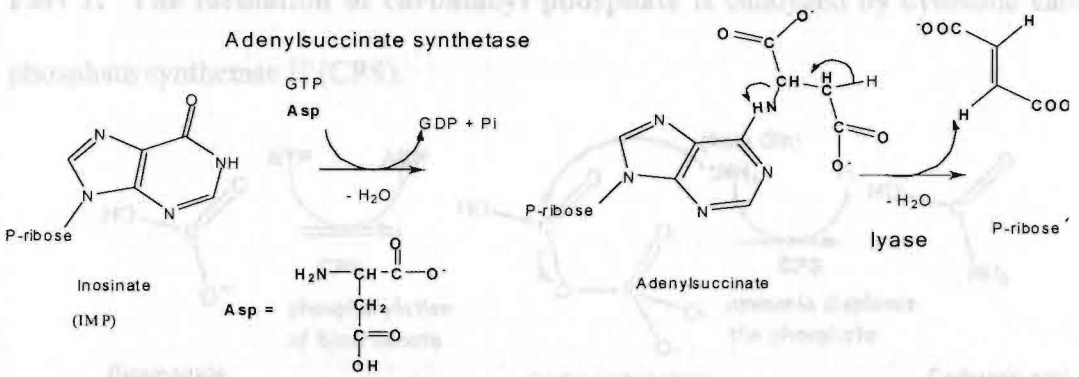


Figure 1-4: Formation of AMP from IMP

Formation of guanylate (GMP): Inosinate is first oxidized to xanthylate, and the C-2 carbonyl is then converted to an amino group:

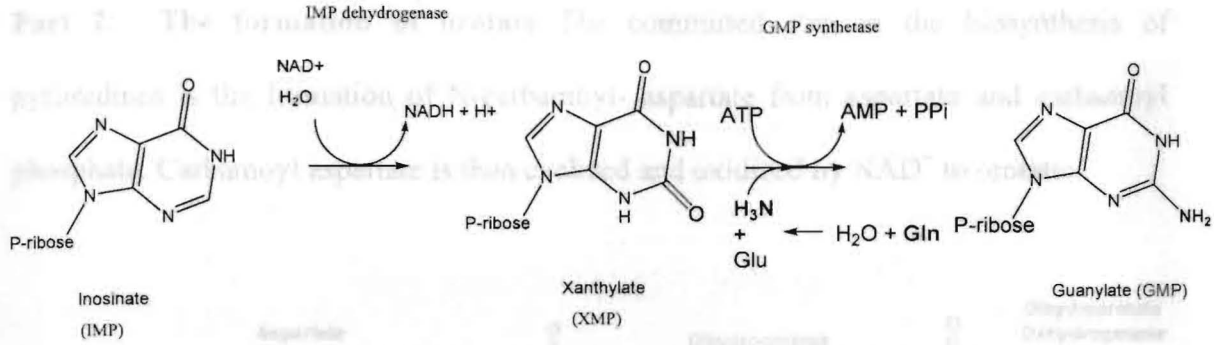


Figure 1-5: Formation of GMP from IMP

***De novo* biosynthesis of Pyridmidines**:

The pyrimidine ring is synthesized in a 6-step process that requires participation of six enzymes. Most required enzymes are cytosolic, with the exception of dihydroorotate dehydrogenase that is localized in mitochondria (see below). The general strategy is to use pre-assembled components (carbamoyl phosphate and aspartate) to make a pyrimidine ring which is then attached to the phosphoribose.[2]

Part 1: The formation of carbamoyl phosphate is catalyzed by cytosolic carbamoyl phosphate synthetase II (CPS).

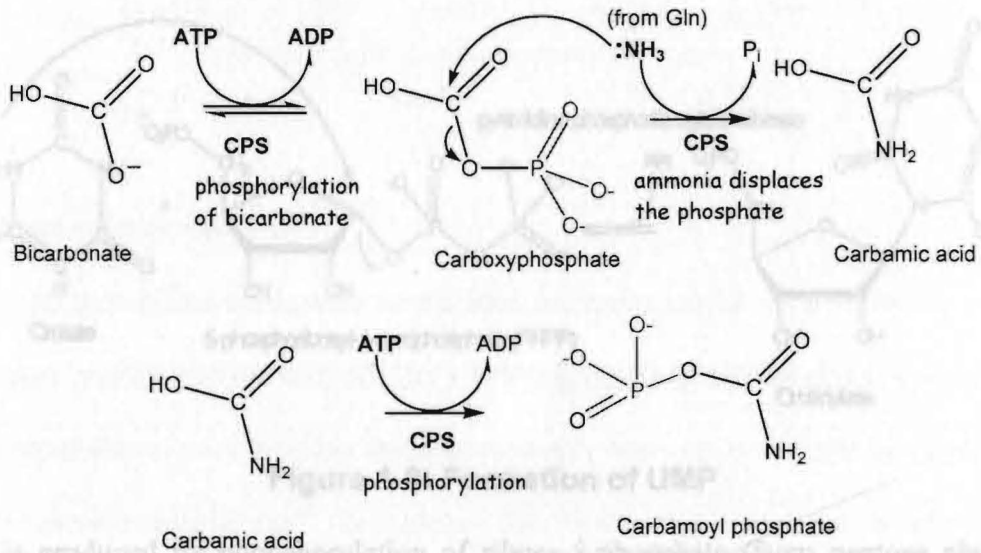


Figure 1-6: Formation of carbamoyl phosphate.

Part 2: The formation of orotate The committed step in the biosynthesis of pyrimidines is the formation of N-carbamoyl- aspartate from aspartate and carbamoyl phosphate. Carbamoyl aspartate is then cyclized and oxidized by NAD^+ to orotate:

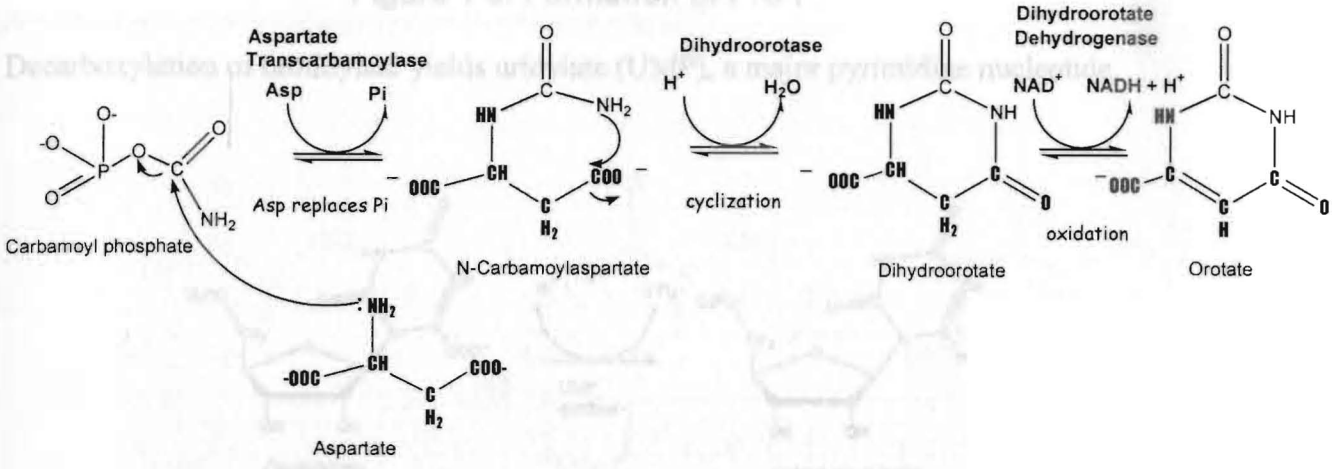


Figure 1-7: Formation of Orotate.

Part 3: Formation of UMP. Orotate coupling to ribose in the form of 5-phosphoribosyl-1-pyrophosphate (PRPP) produces orotidylate (OMP):

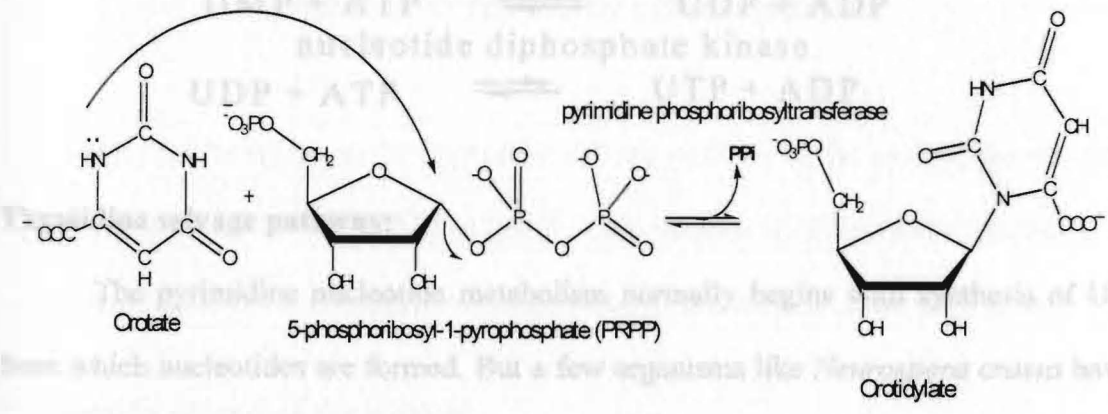


Figure 1-8: Formation of UMP

PRPP is produced by phosphorylation of ribose-5-phosphate (from pentose phosphate pathway):

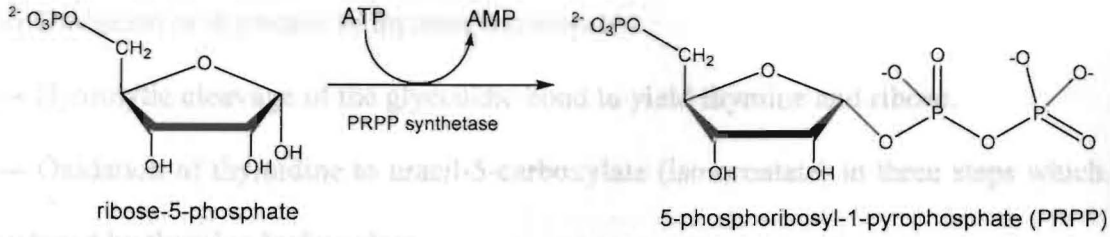


Figure 1-9: Formation of PRPP

Decarboxylation of orotidylate yields uridylate (UMP), a major pyrimidine nucleotide.

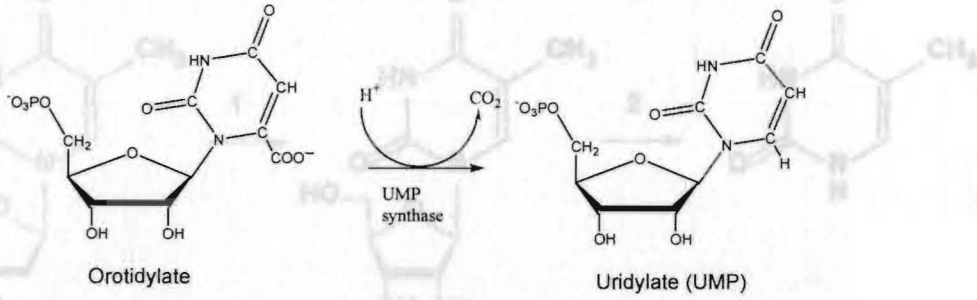


Figure 1-10: Formation of UMP

Phosphorylation of UMP by kinases gives rise to UDP and UTP:

$$\text{UMP} + \text{ATP} \xrightleftharpoons{\text{UMP kinase}} \text{UDP} + \text{ADP}$$

$$\text{UDP} + \text{ATP} \xrightleftharpoons{\text{nucleotide diphosphate kinase}} \text{UTP} + \text{ADP}$$

Thymidine salvage pathway:

The pyrimidine nucleotide metabolism normally begins with synthesis of UMP from which nucleotides are formed. But a few organisms like *Neurospora crassa* have a unique capability of metabolizing thymine to uracil, thus conserving the pyrimidine ring for subsequent metabolic use[3]. The pathway involved in this conversion is referred to as thymidine salvage pathway. The enzymes of this pathway convert thymidine to uracil through the following enzymatic steps.

1→ Oxidation of thymdine to thymine nucleoside.

2→ Hydrolytic cleavage of the glycosidic bond to yield thymine and ribose.

3→ Oxidation of thymidine to uracil-5-carboxylate (iso-oroatate) in three steps which is catalyzed by thymine hydroxylase.

4→ Decarboxylation of iso-orotate to produce uracil.[4]

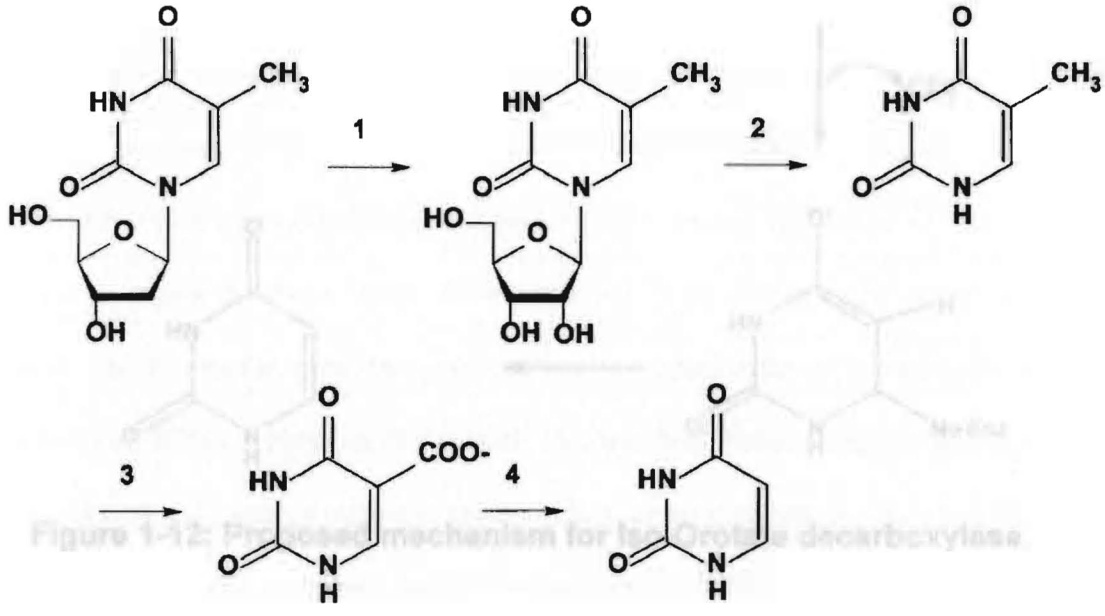


Figure 1-11: Thymidine salvage pathway

The final enzyme in the thymidine salvage pathway is Iso-orotate decarboxylase which converts Iso-orotate (IOA) to uracil. This enzyme is of particular interest to our research group as not a very significant amount of work has been done on it in the past.

Mechanistic aspects of the enzyme:

The mechanism as proposed by Smiley *et al.* involves an attack by a nucleophile in the active site at the 6 position of the iso-orotate, followed by the loss of carboxylate, leaving the enolate of uracil bound to the active site residue. Finally, the enzyme is removed from the substrate, leaving uracil. The non-enzymatic reaction also proceeds in

the same manner. The nucleophile in the case of non-enzymatic reaction is hydroxyl ion. The reaction is proposed to follow the same pattern as that of a non-enzymatic reaction.

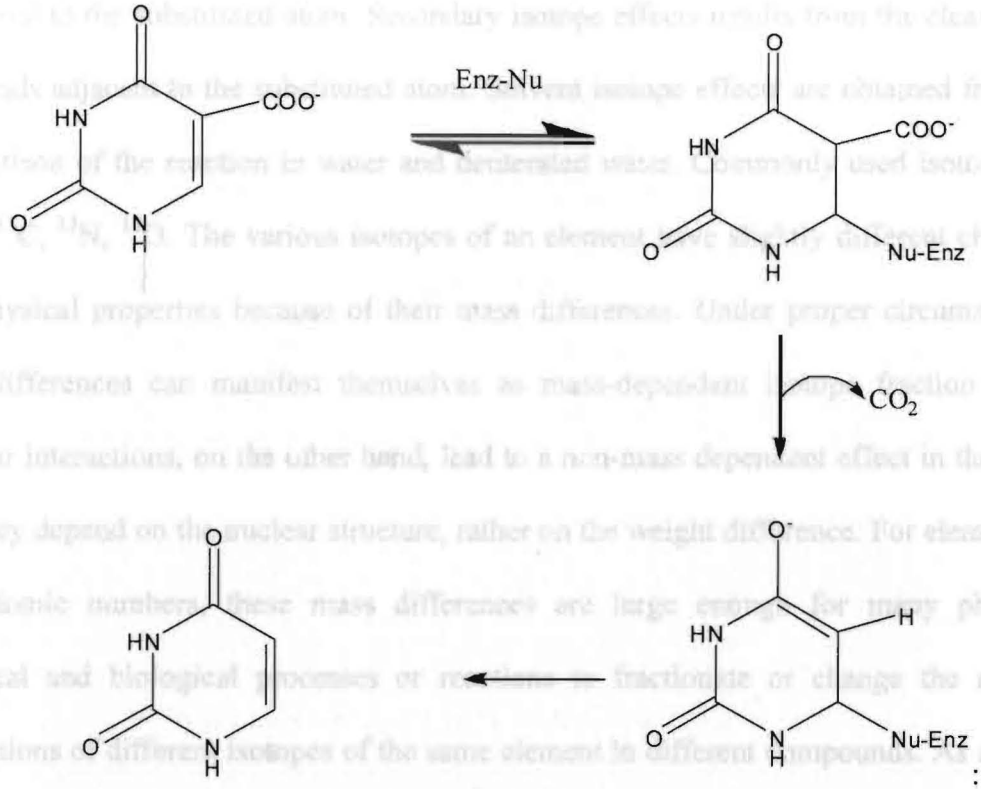


Figure 1-12: Proposed mechanism for Iso-Orotate decarboxylase.

As mentioned above this enzyme is involved in a decarboxylation reaction. There are four main types of decarboxylation reactions: α-keto-acid, β-keto-acid, amino acids, and oxidative decarboxylations[5]. Though it is not one of the types of the decarboxylases mentioned above, a possible mechanism of the IDCase resembles a β-keto acid decarboxylation after a proposed conjugate addition. Several pyrimidine-metabolizing enzymes, such as thymidylate synthase, make this type of nucleophilic addition. Therefore, this provides a unique and interesting enzyme to study.

This research mainly concentrated on the use of isotopes to gain mechanistic information in both enzymatic and non-enzymatic reactions. The effects of isotopes can play a role in determining the mechanistic aspects of a reaction. There are three types of

isotope effects commonly employed in the study of enzymes. These are primary, secondary and solvent isotope effects. A primary isotope effect results from the cleavage of a bond to the substituted atom. Secondary isotope effects results from the cleavage of the bonds adjacent to the substituted atom. Solvent isotope effects are obtained from the comparison of the reaction in water and deuterated water. Commonly used isotopes are D, T, ^{13}C, ^{15}N, ^{18}O. The various isotopes of an element have slightly different chemical and physical properties because of their mass differences. Under proper circumstances, such differences can manifest themselves as mass-dependent isotope fraction effect. Nuclear interactions, on the other hand, lead to a non-mass dependent effect in the sense that they depend on the nuclear structure, rather on the weight difference. For elements of low atomic numbers, these mass differences are large enough for many physical, chemical and biological processes or reactions to fractionate or change the relative proportions of different isotopes of the same element in different compounds. As a result of fractionation processes, waters and solutes often develop unique isotopic compositions (ratios of heavy to light isotopes) that may be indicative of their source or the process that formed them. Two main types of phenomena produce isotopic fractionations: isotopic exchange and kinetic process. The effects of isotopes on the kinetics of a reaction depend on the size of the isotope and the type of reaction it is involved in. If the reaction proceeds through nuclear interactions the binding effects are changed and it is this change that can be measured. The use of isotope labeling for enzyme kinetics is widely used and simple to employ. The use of isotope labeling method will in part assist in the determination of the mechanism used by the protein. The following experiments have been designed in this thesis:

1) The study of kinetics of the reaction catalyzed by Iso-Orotate decaroxylase.

2) The study of isotope effects involved in the reaction which will help in identifying the mechanistic aspects of the reaction.

Chapter 2

Kinetic studies on conversion of Iso-Orotate to uracil

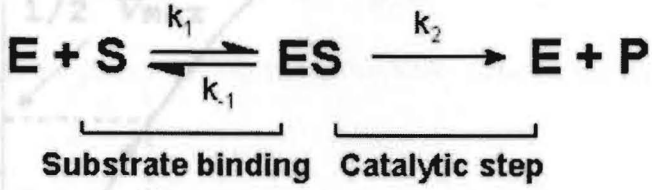
Enzymes are usually proteins of high molecular weight (15,000 < MW < several million Daltons) that acts as catalysts. Enzymes are versatile and very effective biological catalysts. Enzymes are substrate specific and are classified according to the reaction they catalyze. The reaction rates under ambient conditions are much higher when compared to chemically catalyzed reactions. Enzymes are named by adding suffix *–ase* to the end of the substrate or the reaction catalyzed. Enzymes that occur in several different forms, but catalyze the same reaction, are called *isozymes*[6]. Enzyme catalysis is affected by primary, secondary, tertiary and quaternary structure of the enzymes; such enzymes require a non protein group for their activity. This nonprotein group is either a cofactor, such as metal ions, Mg, Zn, Mn, etc., or a coenzyme, such as organic molecule, NAD, FAD and some vitamins. Enzyme activity is also affected by inhibitors, temperature, pH and concentration of the substrate.

Enzymes do not affect the free-energy change or the equilibrium constant. They lower the activation energy of the reaction by binding the substrate and forming enzyme-substrate [ES] complex. The interaction between the enzyme and its substrate is usually by weak forces such as van der Waals forces and hydrogen bonding. The substrate binds to a specific site on the enzyme called *active site*[7]. More of the interactions occur in the transition state because of the structured active site.

Enzyme Kinetics:

Enzyme Kinetics[8] is the study of how enzymes bind substrates and turn them into products. This can be done by studying the impact made on the rate of an enzyme – catalyzed reaction by changing the experimental conditions. The main concept in the

enzyme kinetics is the effect of substrate concentration on the initial rate of an enzyme catalyzed reaction.



Michaelis –Menten Kinetics

In 1913 Leonor Michaelis and Maud Menten proposed a quantitative relationship between substrate and enzyme in an enzymatic reaction. This kinetic model is valid only when the concentration of enzyme is much less than the concentration of substrate (i.e., enzyme concentration is the limiting factor), and when the enzyme is not allosteric.

The Michaelis-Menten equation[9] is the rate equation for a one-substrate enzyme-catalyzed reaction. It quantitatively relates the initial rate (V_0), the maximum rate (V_{max}) and the initial substrate concentration **[S]** to the Michaelis constant K_m.

$$V_0 = V_{(max)} [S] / (K_m + [S])$$

Maximum Velocity [$V_{(max)}$]:

The maximum rate of an enzyme mediated reaction is determined by increasing the substrate concentration [S] until a constant rate of product formation is achieved at optimal pH and temperature. This is the maximum velocity (V_{max}) of the enzyme. At this stage the enzyme sites are saturated with the substrate. (V_{max}) is never achieved in reality, because at this stage all enzyme molecules must be tightly bound with substrate. So, the characteristic value of (V_{max}) for the enzyme is defined by the substrate concentration at its half-maximum speed i.e., (V_{max}) / 2.

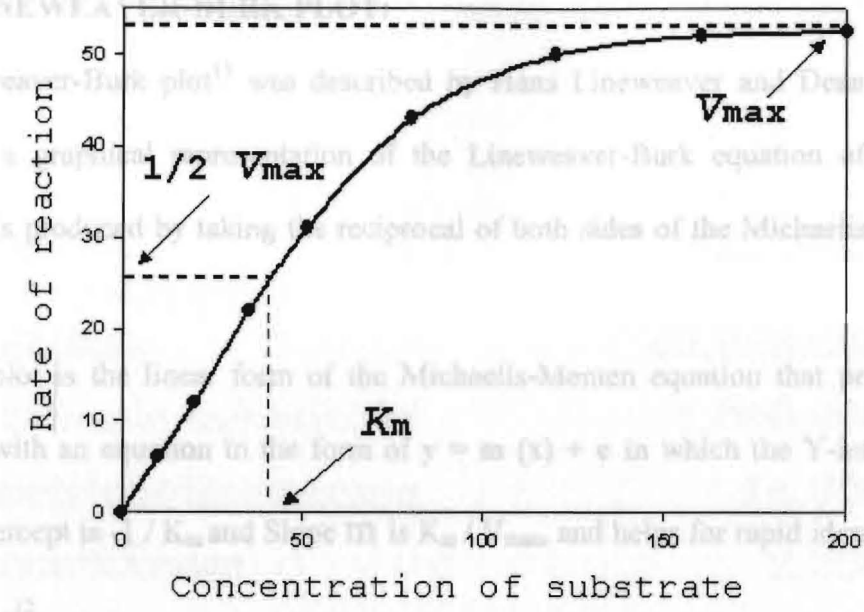


Figure 2-1: Saturation plot to obtain [S] and V.
Source:http://en.wikipedia.org/wiki/Image:MM_curve_v2.png

The speed V can be obtained by determining the number of reactions per second that are catalyzed by an enzyme.

Michaelis constant (K_m):

The Michaelis constant[5] is the substrate concentration at which the reaction occurs at half of the maximum rate i.e., $(V_{max}) / 2$. It indicates the affinity of an enzyme towards a given substrate which implies the stability of the enzyme-substrate complex. Small K_m values indicate the tight binding of enzyme molecules with substrate and high K_m values indicate weak binding of enzyme molecules with substrate. There are limitations in the quantitative interpretation of Michaelis plot.

As the (V_{max}) is never achieved in reality, the values of (V_{max}) and K_m are approximately calculated from the Michaelis plot. By converting the data into a linear Lineweaver-Burk plot or Eadie-Hofstee plot the values of (V_{max}) and K_m are accurately determined (though still not perfect).

LINEAR LINEWEAVER-BURK PLOT:

Lineweaver-Burk plot[11] was described by Hans Lineweaver and Dean Burk in 1934. It is a graphical representation of the Lineweaver-Burk equation of enzyme kinetics that is produced by taking the reciprocal of both sides of the Michaelis–Menten equation.

This plot is the linear form of the Michaelis-Menten equation that produces a straight line with an equation in the form of **y = m (x) + c** in which the Y-intercept is $1/V_{max}$, X-intercept is $-1 / K_m$ and Slope **m** is K_m / V_{max}, and helps for rapid identification of these values[12].

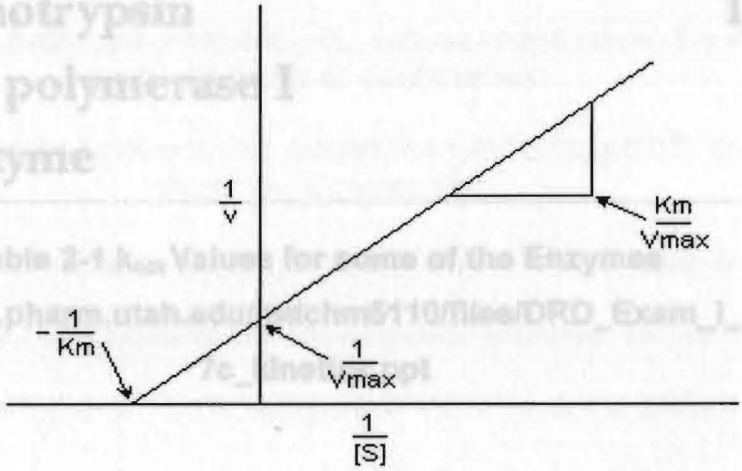


Figure 2-2: Linear Line weaver-Burk plot.
Source: http://en.wikipedia.org/wiki/Image:Lineweaver-Burke_plot.PNG

There is no possibility of taking negative experimental values of substrate concentration. So, X-intercept is just the extrapolation of the experimental data taken at finite substrate concentration.

The efficiency of an enzyme can be expressed in terms of k_{cat}/K_m. When the substrate concentration is very high then the catalytic rate is equal to $k_{cat.}$, which is termed as turnover number. The turnover number is defined as the maximum number of moles

of substrate that an enzyme can convert to product per catalytic site per unit time. The turnover numbers of some of the enzymes are as follows:

Enzyme	k_{cat} (sec^{-1})
Catalase	40,000,000
Carbonic anhydrase	1,000,000
Acetylcholinesterase	14,000
Penicillinase	2,000
Lactate dehydrogenase	1,000
Chymotrypsin	100
DNA polymerase I	15
Lysozyme	0.5

Table 2-1 k_{cat} Values for some of the Enzymes

Source:http://rx1.pharm.utah.edu/mdchm5110/files/DRD_Exam_I_II/lecture_7c_kinetics.ppt

As explained earlier the catalytic efficiency is given by k_{cat}/K_m values. These values give an indication of how perfect an enzyme is. k_{cat}/K_m is an apparent second-order rate constant. It measures how efficiently an enzyme performs when substrate concentration is low. The upper limit for k_{cat}/K_m is the diffusion limit, i.e., the rate at which E and S diffuse together (10^8 to 10^9 s^{-1}, at ambient temperatures in water). The examples of some of the enzymes whose k_{cat}/K_m values approaches the diffusion controlled rate of association with substrate are as follows:

Enzyme	Substrate	k_{cat} (sec^{-1})	K_m (M)	k_{cat}/K_m ($sec^{-1} M^{-1}$)
Acetylcholinesterase	Acetylcholine	1.4×10^4	9×10^{-5}	1.6×10^8
Carbonic anhydrase	CO_2	1×10^6	0.012	8.3×10^7
	HCO_3^-	4×10^5	0.026	1.5×10^7
Catalase	H_2O_2	4×10^7	1.1	4×10^7
Crotonase	Crotonyl-CoA	5.7×10^3	2×10^{-5}	2.8×10^8
Fumarase	Fumarate	800	5×10^{-6}	1.6×10^8
	Malate	900	2.5×10^{-5}	3.6×10^7
Triosephosphate isomerase	Glyceraldehyde-3-phosphate*	4.3×10^3	1.8×10^{-5}	2.4×10^8
β-Lactamase	Benzylpenicillin	2×10^3	2×10^{-5}	1×10^8

Table 2-2 Enzymes whose k_{cat}/K_m values approaches the diffusion controlled rate of association.

Source:http://rx1.pharm.utah.edu/mdchm5110/files/DRD_Exam_I_II/lecture_7c_kinetics.ppt

The complete understanding of the mechanism of Iso-Orotate decarboxylase involves the study of both enzymatic and non-enzymatic reactions. The study of kinetics of both enzymatic and non-enzymatic will provide a clue about the mechanistic aspects of the enzyme. The mechanism as proposed by Smiley *et al.* involves an attack by a nucleophilic residue in the enzyme at the 6 position of the iso-orotate, followed by the loss of carboxylate, leaving the enolate of uracil bound to the active site residue. Finally, the enzyme is removed from the substrate, leaving uracil. The non-enzymatic reaction is also proposed to proceed in the same manner. The mechanism is also believed to depend on the pH of the environment. In acidic environment the CO_2 group in fifth position of the 5-carboxy- uracil will be protonated and therefore is not a good leaving group. In basic environment the nitrogen in position 1 is in ionic state and the molecule is not

suitable for nucleophilic attack at C-6. When the conditions are right and the pH is suitable then the nucleophilic attack starts at C-6 starting the decarboxylation reaction.

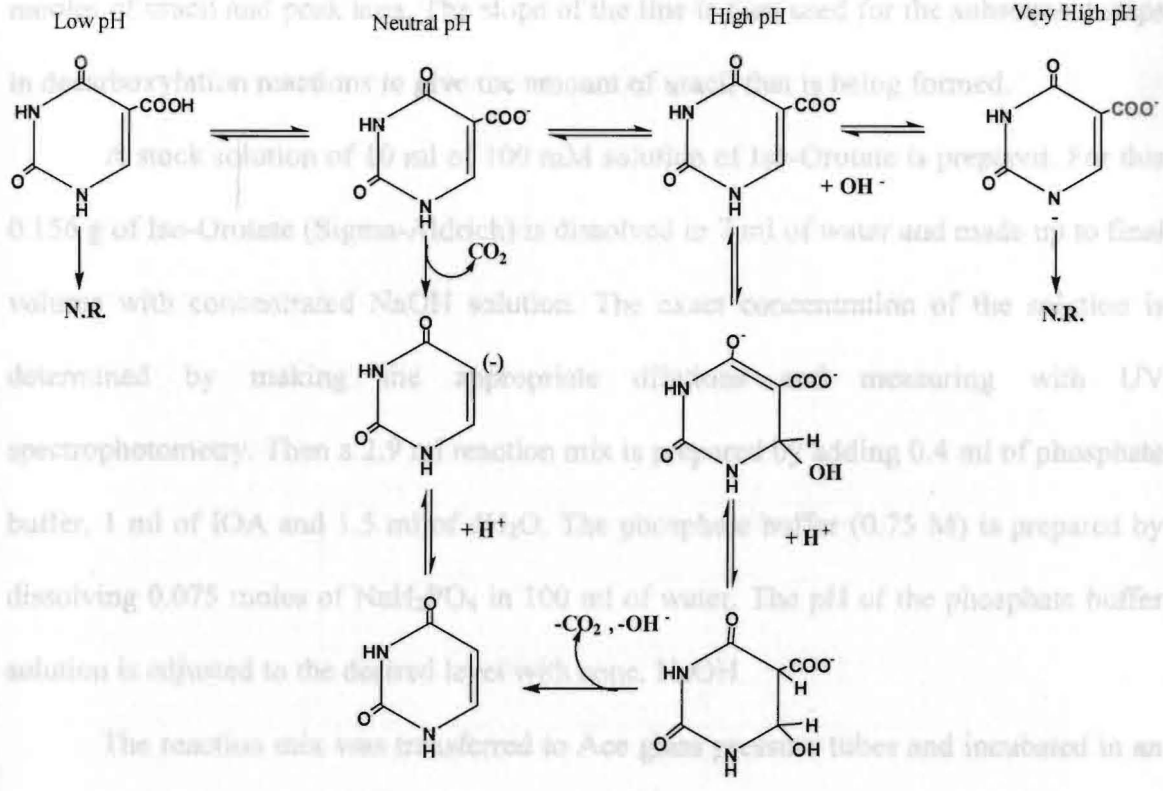


Figure 2-3: Proposed mechanism for the non-enzymatic decarboxylation of iso-orotate.

To get complete understanding of the mechanistic aspects of iso-orotate decarboxylase, kinetic measurement of the non-enzymatic reaction have been planned. These experiments are aimed at eventually studying the efficiency of the enzyme as well as to get a good understanding of the mechanism involved.

Materials and Methods:

A uracil standard curve is prepared with the help of UV Spectrometry and HPLC. A sample of 78 mg of uracil (Sigma-Aldrich) is dissolved in water and NaOH and the absorbances of diluted samples are measured at 260 nm. After determining the concentrations, known amounts of uracil are then injected into the HPLC system. The

volumes of injections normally range from 5 µl to 50 µl. The retention time of uracil is then determined. The peak areas corresponding to various concentrations are then determined and plotted on a graph. Then a linear relationship is obtained between the nmoles of uracil and peak area. The slope of the line is then used for the subsequent steps in decarboxylation reactions to give the amount of uracil that is being formed.

A stock solution of 10 ml of 100 mM solution of Iso-Orotate is prepared. For this 0.156 g of Iso-Orotate (Sigma-Aldrich) is dissolved in 7 ml of water and made up to final volume with concentrated NaOH solution. The exact concentration of the solution is determined by making the appropriate dilutions and measuring with UV spectrophotometry. Then a 2.9 ml reaction mix is prepared by adding 0.4 ml of phosphate buffer, 1 ml of IOA and 1.5 ml of dH_2O. The phosphate buffer (0.75 M) is prepared by dissolving 0.075 moles of NaH_2PO_4 in 100 ml of water. The pH of the phosphate buffer solution is adjusted to the desired level with conc. NaOH.

The reaction mix was transferred to Ace glass pressure tubes and incubated in an oil bath at 100°C. The non-enzymatic conversion of Iso-Orotate to uracil is very slow and so the reaction is normally carried at temperatures as high at 100°C to increase the speed of the reaction. Periodic checks for the formation of uracil from Iso-Orotate are made by removing small aliquots after regular time intervals, analyzing by HPLC, and comparing the uracil peak to the standard curve of uracil. The reaction time is stopped each time the reaction tube is removed from the oil bath. The reactions are run at various pH's from 7.18 to 12.94. Lower pH was not utilized in this procedure due to the lower solubility of iso-orotate and uracil.

The samples that are removed from reaction tubes are used to calculate the amount of uracil that is being formed and the amount of Iso-Orotate that is unreacted. The peak areas are calculated by using Waters HPLC system and the Breeze software.

The isocratic pump method is followed with a flow rate of 1 ml/minute. A buffer solution consisting of 4:1 mixture of 10 mM potassium phosphate at pH 2.5 and CH_3CN is used as a solvent for the HPLC system. The uracil is found to have a retention time of about 8 minutes and Iso-Orotate is found to have a retention time of about 13 minutes. The aliquots that are removed from the reaction tubes at various pH's are injected in to the HPLC system. The concentration of uracil is then determined by comparing to the standard curve of uracil. The concentration of uracil that is formed gives the remaining molar concentration of IOA. The rate of reaction (k) value can be determined by plotting natural log of the remaining IOA concentration against the reaction time.

Results and Discussion

Conc. of uracil (nmol)	Peak area
22	1071638
44	3981929
66	8483970
88	10046887
110	14081721

Table 2-3: The table gives peak areas corresponding to various amounts of uracil. This data is used to construct a linear plot between nmoles of uracil and peak area which is necessary for kinetic studies.

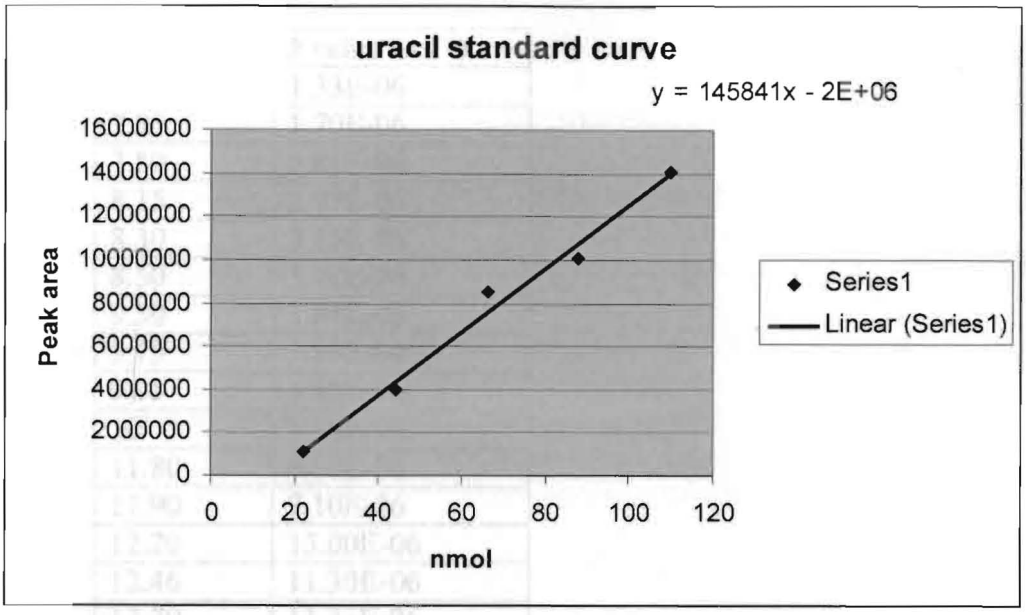


Figure 2-4: Standard curve of uracil

Calculation of rate constant from HPLC data:

The samples that are removed from reaction at regular intervals are injected in to HPLC system and the corresponding concentration of uracil is detected from the uracil standard curve. From this the amount of Iso-orotate that is remaining can be calculated. A graph is plotted between the natural log of remaining concentration of Iso-orotate and time. The slope of the graph gives is negative, but the rate constant is the negative value of the slope and therefore positive.

pH	*k* value (sec^{-1})
7.18	1.33E-06
7.25	1.70E-06
7.80	2.81E-06
8.15	2.97E-06
8.30	3.15E-06
8.50	3.42E-06
9.50	3.84E-06
9.75	3.89E-06
9.95	4.60E-06
10	3.60E-06
11.80	6.90E-06
11.90	7.10E-06
12.20	13.00E-06
12.46	11.30E-06
12.70	11.31E-06

Table 2-4: Rate constants (*k*) for the decarboxylation of iso-orotate measured at different pH's.

The data gives an indication of the mechanistic aspects of the non-enzymatic decarboxylation of Iso-Orotate. The rate constant increased with increased in pH. The rate decreased at pH 10. The rate increased appreciably at pH 12.2. The rate decreased at pH higher than 12.2

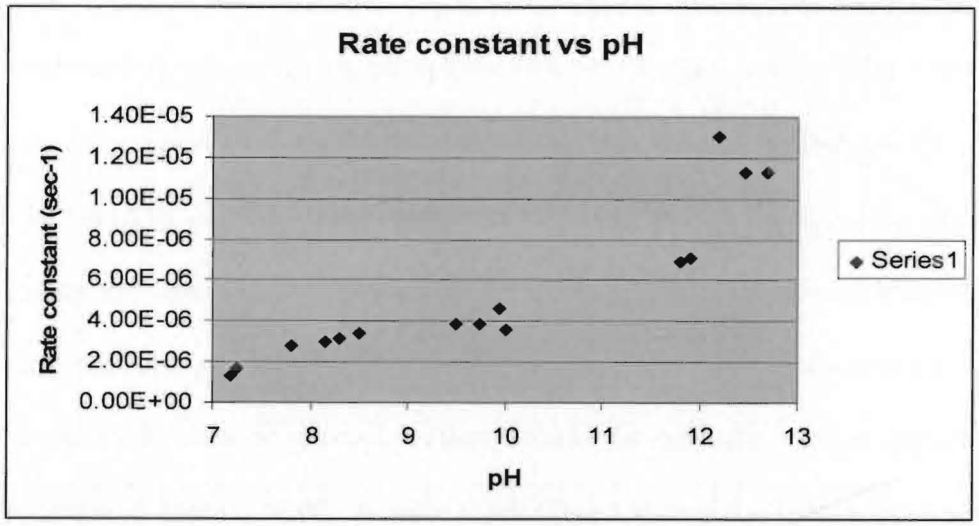


Figure 2-5: Graph of combined k values from the non-enzymatic reaction (The complete data can be seen in Appendix A)

The above graph explained relationship between pH and non-enzymatic rate constants for the conversion of Iso-Orotate to uracil. From the graph it is evident that rate of decarboxylation increased with increase in pH. The decrease in rate constant at pH 10 needs to be studied further. The optimum pH for rate of decarboxylation is pH 12.2. The data supports the proposed mechanism for non-enzymatic reaction. As the concentration of the nucleophile increased the rate of conversion from Iso-Orotate increased. The rate of reaction decreased above the pH of 12.2 which might be attributed to the presence of excessive OH ion which causes the deprotonated species of IOA, unfavoring decarboxylation.

Chapter 3

Isotope effects

The effect on the rate or equilibrium constant of two reactions that differ only in the isotopic composition of one or more of their otherwise chemically identical components is referred to as an isotope effect.[13] There are three types of isotope effects commonly employed in the study of enzymes. These are primary, secondary and solvent isotope effects. A primary isotope effect results from the cleavage of a bond to the substituted atom. Secondary isotope effects results from the cleavage of the bonds adjacent to the substituted atom. Solvent isotope effects are obtained from the comparison of the reaction in water and deuterated water. Commonly used isotopes are D, T, ^{13}C, ^{15}N, and ^{18}O. The various isotopes of an element have slightly different chemical and physical properties because of their mass differences. Under proper circumstances, such differences can manifest themselves as mass-dependent isotope fraction effect.

Isotope effects are caused by substituting a heavy isotope for the normal lighter one. Kinetic isotope effects are on the rates while equilibrium isotope effects are on the equilibrium of a reaction. Equilibrium isotope effects are determined by the relative stiffness of bonding of the isotopic atom in substrate and product, while the kinetic isotope effects are determined by the stiffness of bonding in substrate and transition state [14]. In case of equilibrium isotope effect the heavy isotope gets enriched in the more stiffly bonded position. In case of kinetic isotope effects the primary isotope effects where bonds are made or broken to the isotopic atom always shows discrimination against the heavy isotope. This produces a normal isotope effect since the isotope effects are expressed as ratio of rate for the light isotope to that for the heavy one. Secondary isotope

effects where no bonds are made or broken to the isotopic atom can be normal one way and inverse (less than one) the other. A leading subscript is used to indicate the nature of isotopic effect, with D, T, 13, 14, 15 or 18 indicating deuterium, tritium, ^{13}C, ^{14}C, ^{15}N, ^{18}O substitution. Thus

$$^{D}k = k_H/k_D \text{ and } ^{13}K_{eq} = K_{eq\ c12}/K_{eq\ c13}.$$

In most enzymatic reactions the rate of reaction is given by:

$$v = VA / (K+A)$$

Where A is the substrate concentration, V is the maximum velocity, and K is the Michaelis constant of A. The independent kinetic constants are V and V/K, with V/K being the apparent first-order rate constant at low substrate concentrations. Thus, the isotope effects are determined on V and V/K.

There are three methods for determining the isotopic effects on enzymatic reactions. The simplest method is a comparison of reciprocal plots with labeled and unlabeled substrates. The ratio of slopes is the V/K isotope effect and the ratio of vertical intercepts is the v isotopic effect. This method is limited to deuterium isotope effects larger than 1.05. The second method is equilibrium perturbation. [15] In this case a reaction mixture close to equilibrium is set up with a labeled reactant on one side of the reaction and an unlabeled one on the other side. The reaction moves away from the equilibrium once the reaction is initiated, as the unlabeled one reacts faster than the labeled one. The reaction finally returns to both chemical and isotopic equilibrium as isotopic mixing occurs. The method works well if one of the reactant is colored. The molecules between which the label is exchanged are called perturbants, and the fractional perturbation from equilibrium is calculated. This method is believed to measure isotopic effects as low as 1.03. But the values are similar to isotope effects on V/K and give no information on V. The third method for measuring isotopic effects is internal competition. In this method

the changes in the mass ratio or specific activity of substrate or product as the reaction proceeds is measured. This is usually followed for tritium and carbon isotope effects.

Isotope effects as small as 1.002 can be measured with an isotope ratio mass spectrometer, which can be used to determine changes in ^{13}C, ^{15}N or ^{18}O mass ratios in CO_2 or N_2. This method determines the isotope effect on V/K for the labeled substrate[16]. If R_O is the specific activity or isotope ratio of initial substrate to product at 100% reaction, R_p is the specific activity or isotope ratio of product at fraction of reaction *f*, and R_s is the specific activity or isotope ratio of residual substrate at fraction of reaction *f* then:

$$^{T}(V/K) = \log (1-f)/\log (1-f\, R_P/R_0)$$

$$^{T}(V/K) = \log (1-f)/\log [(1-f)\, (R_S/R_0)]$$

Two main types of phenomena produce isotopic fractionations: isotopic exchange and kinetic process. The effects of isotopes on the kinetics of a reaction depend on the size of the isotope and the type of reaction it is involved in. If the reaction proceeds through nuclear interactions the binding effects are changed and it is this change that can be measured [17]. The use of isotope labeling for enzyme kinetics is widely used and simple to employ. The use of isotope labeling method will in part assist in the determination of the mechanism used by the protein. The typical kinetic isotope effect values for some of the elements are given below:

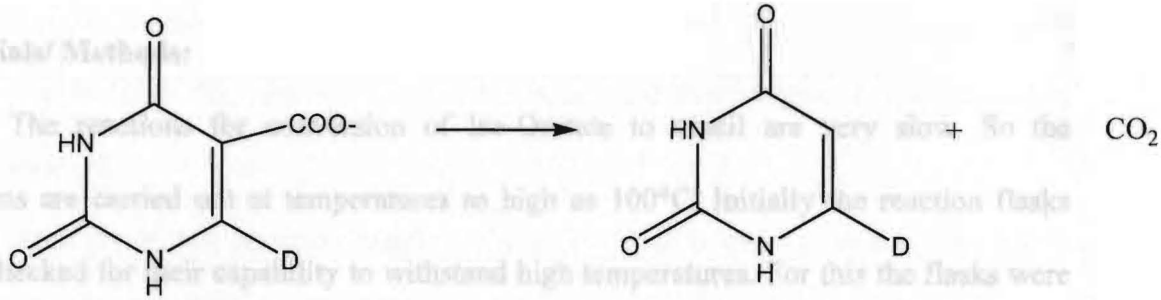
Nuclide	*k* (light) / *k* (heavy)
C-H/C-D	6-8
C-H/C-T	15-16
$^{12}C/^{13}C$	1.06
$^{12}C/^{14}C$	1.10
$^{14}N/^{15}N$	1.03
$^{16}O/^{18}O$	1.02
$^{32}S/^{34}S$	1.01
$^{35}Cl/^{37}Cl$	1.01

Table 3-1: Isotpe effects of elements

The technique of isotope effects to study mechanistic aspects of enzymes is expanding every day. There have been few recent additions to the technical methods employed in the study of isotope effects. These designs include the use of isotope effects to determine kinetics of a reaction, distinguish between reactant–state origins and transition-state origins, distinguish between concerted and stepwise chemical mechanisms, characterize bond order changes in ligand binding, distinguish different pathways of inhibitor binding, and estimate intrinsic isotope effects.[18] These isotope effects can be effectively used to study the reaction catalyzed by Iso-Orotate decarboxylase. The study of secondary isotope effects and carbon isotope effects will help in determining the mechanism involved in conversion of Iso-Orotate to uracil.

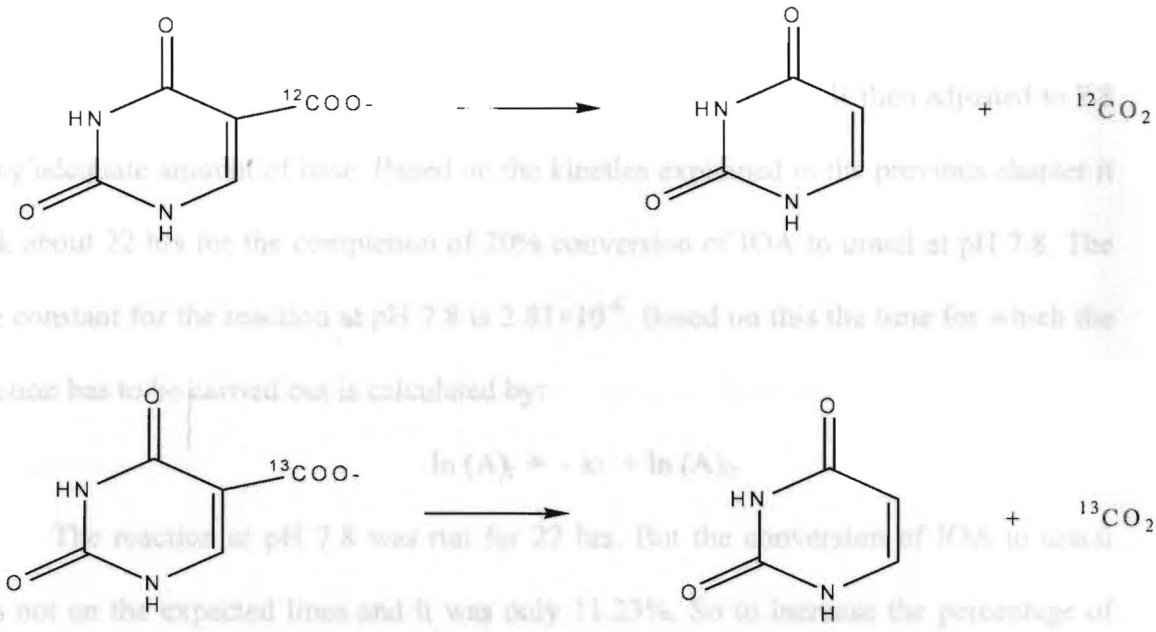
Study of secondary hydrogen isotope effects:

This involves the synthesis of ^{2}H6 enriched iso-orotate from ^{2}H6 uracil. The rate of the reaction is measured and compared with rate of reaction with 1H6 iso-orotate. The ratio of the rates gives the secondary hydrogen isotope effect. The typical effect of the deuterium isotope effect is about 1.5. This means that by adding the deuterium in such a way as to ensure that the proposed mechanism has to operate through the labeled bond and thereby producing a change in the rate of reaction. If the nucleophile proceeds as proposed then the deuterium should slow down the rate of reaction.



Carbon isotope effects:

For measuring the carbon isotope effects decarboxylation reactions are run to 100% completion and to partial (around30%). Carbon dioxide is isolated in both cases and isotope content is measured. The isotope effect ($^{12}k/^{13}k$) is calculated from the two isotope ratios. The greatest possible isotope effect is around 1.06. If the measured isotope effect is significantly less than 1.06(1.01-1.02), it indicates that a covalent step took place before decarboxylation.



Materials/ Methods:

The reactions for conversion of Iso-Orotate to uracil are very slow. So the reactions are carried out at temperatures as high as 100°C. Initially the reaction flasks were checked for their capability to withstand high temperatures. For this the flasks were placed in oil bath for overnight. Bicarbonate solution was placed in the flask and the collection of carbon dioxide was done with this standard solution. First the standard carbon dioxide gas obtained from cylinder is analyzed using IRMS. Then the standard bicarbonate solution is degassed using an inert gas and then subjected to collection of carbon dioxide. Then the carbon dioxide gas was analyzed using the standard gas as reference. The collection of carbon dioxide was found to be normal with the flasks and the delta value obtained from IRMS was close to the collections done with the earlier standard bicarbonate solutions. So it was concluded that the flasks were able to with stand high temperatures and further analysis was carried using the flasks.

Procedure for partial reaction:

The reactions were carried out using at pH 7.8. For this 10 mM IOA is prepared and mixed with adequate buffer solution. To prepare 10 mM Solution of Iso-Orotate

0.156 g of Iso-Orotate is dissolved in 10 ml of water. The reaction mix is prepared by mixing 5ml of 10 mM IOA with 5 ml of phosphate buffer. The pH is then adjusted to 7.8 using adequate amount of base. Based on the kinetics explained in the previous chapter it took about 22 hrs for the completion of 20% conversion of IOA to uracil at pH 7.8. The rate constant for the reaction at pH 7.8 is 2.81×10^{-6}. Based on this the time for which the reaction has to be carried out is calculated by:

$$\ln (A)_t = - kt + \ln (A)_0$$

The reaction at pH 7.8 was run for 22 hrs. But the conversion of IOA to uracil was not on the expected lines and it was only 11.23%. So to increase the percentage of conversion the reaction has been run for 46 hrs. This showed the percentage conversion of uracil to be 23%. The reaction was run for 46 hrs and then subjected to collection of carbon dioxide.

After the 46 hrs, adequate amount of sulphuric acid is added to release the carbon dioxide that is dissolved in the solution. The carbon dioxide that is collected is then analyzed using IRMS. The delta value is then noted.

Procedure for complete reaction:

The complete conversion from IOA to uracil is done using Iso-Orotate decarboxylase enzyme. The IDCase enzyme which was earlier purified by Ragini Kankanala and which is supposed to have the activity of 1nm/µg/min is used. For complete conversion reaction 1 ml of 10 mM solution of IOA is taken and mixed with 9 ml of phosphate buffer solution. The pH is then adjusted to 7.8. To this 1 ml of enzyme is added. The reaction is allowed for one hour. This is expected to convert all the Iso-Orotate to uracil. The solution is then degassed for about one hour. After degassing 2 ml of 100 mM sulfuric acid is added to the solution and subjected to collection of carbon

dioxide. Then the carbon dioxide that is collected is analyzed using IRMS and the delta value is noted.

Results/Discussion:

The CO_2 that is collected from partial reactions and complete reactions are analyzed using a GV dual inlet Isotope Ratio Mass Spectrometer. The reference carbon dioxide is obtained from Praxair. The ^{13}C kinetic isotope effects were calculated using the following equation:

$$KIE = [\log (1-f)] \div \log[1-f\times(Rp/Rs)]$$

Where,

f = fraction of reaction (between 0.0 and 1.0)

Rp = isotope ratio of the product which is determined from the partial reaction.

Rs = isotope ratio of the substrate which is determined from the complete reaction.

Delta values obtained from mass spectrometer are converted in to isotope ratios Rp and Rs as follows:

$$Rp= 1000 + \delta p;\ Rs= 1000+\delta s.$$

The isotope effect values calculated at pH 7.8 is tabulated below:

pH 7.8	Fraction of reaction	Delta value (partial reaction)	Delta value (complete reaction)	Isotope effect
Trial 1	0.24	2.279	21.855	1.0225
Trial 2	0.31	3.521	21.900	1.0221
Trial 3	0.28	2.301	21.000	1.0223
Trial 4	0.27	2.229	20.990	1.0220
Trial 5	0.28	2.301	21.100	1.0220
Trial 6	0.29	2.399	21.100	1.0222
Trial 7	0.32	3.510	21.700	1.0221
Trial 8	0.22	2.800	23.000	1.0229
Trial 9	0.33	3.600	22.000	1.0225
Trial 10	0.29	3.600	22.200	1.0221

Table 3-2: Isotope effect values for different trials at pH 7.8.

The isotope effects calculated at pH 7.8 are very consistent and are around 1.022. Since they are less than the normal value of 1.06, it gives strength to the proposed mechanism that a covalent step involving a nucleophile took place before decarboxylation. The HPLC chromatograms and the isotope effect calculation sheets are provided in appendix B.

Chapter 4

Conclusions

The kinetic studies on the enzyme Iso-orotate decarboxylase has been a rewarding experience for me. Although the kinetic studies are bit complex, they are very important tools for studying the mechanistic aspects of an enzymatic reaction. The conversion of Iso-orotate to uracil is normally very slow at room temperatures and so it has been a challenge for me to run the reaction constantly at elevated temperatures. The kinetic studies done in the lab successfully proved the involvement of a nucleophile prior to decarboxylation step which has been proposed earlier.

Apart from this the work done on isotope effects has been very challenging for me. The results for the isotope effects at pH 7.8 have been very consistent and also prove the fact that a covalent step took place before decarboxylation. But there was a problem with the higher pH's as the results were not consistent. The main problem at higher pH's might be with the degassing of the sample. So if this problem can be overcome then the isotope effect calculations can be done at higher pH. This would render more credibility to the results and can be useful in understanding the mechanistic details of this unique enzyme. Thus this work will pave way for future mechanistic studies on this enzyme.

The work done on non-enzymatic reactions suggested that hydroxyl ion might act as a nucleophile in these decarboxylation reactions. This might also hold good for the enzymatic reaction catalyzed by Iso-orotate decarboxylase. Decarboxylases like ACMSD and Iso-orotate decarboxylase have sequence similarity to a group of demainase subgroup of the amidohydrolase superfamily[19]. While these hydrolytic members of the amidohydrolase superfamily cleave the bond, the above mentioned decarboxylases might use the hydroxyl ion to form an intermediate that easily releases carboxyl group to give

the products. Thus this might also provide the evidence for the involvement of hydroxyl ion as a nucleophile in the reaction catalyzed by Iso-orotate decarboxylase.

Conc. of uracil (nmol)	Peak area
22	1071638
44	3951929
66	8483970
88	10046887
110	14081721

Table A-1: HPLC peak areas from various concentrations of Uracil.

Figure A-1: Uracil standard curve obtained by injecting known amounts of Uracil in HPLC.

Appendix A:

Non Enzymatic kinetic data

Conc. of uracil (nmol)	Peak area
22	1071638
44	3981929
66	8483970
88	10046887
110	14081721

Table A-1: HPLC peak areas from various concentrations of Uracil.

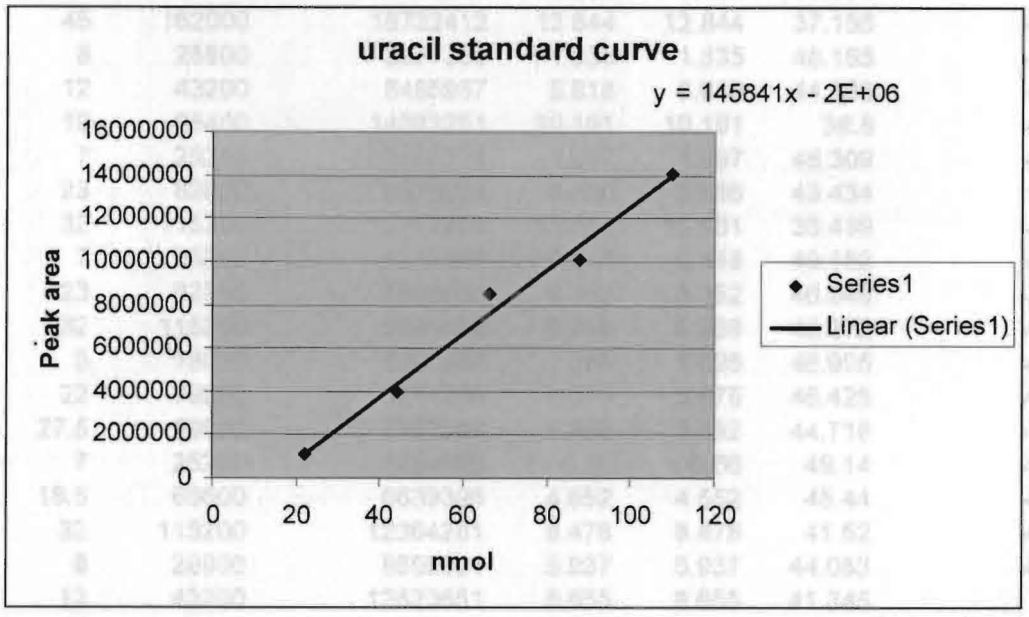


Figure A-1: Uracil standard curve obtained by injecting known amounts of Uracil in HPLC.

Table A-2: Data from non–enzymatic decarboxylation reactions at various pH's

pH	Time(Hrs)	Time(Sec)	Area of Uracil peak	nmol Uracil	[Uracil]	[IOA]	Ln[IOA]t/[IOA]0
7.18	9	32400	32400	0.85	0.85	49.15	-0.017
7.18	22	79200	75600	0.97	0.97	49.03	-0.02
7.18	32	115200	118800	3.67	3.67	46.33	-0.076
7.25	8	28800	3948539	2.707	2.707	47.293	-0.055
7.25	12	43200	12638256	8.657	8.657	41.343	-0.19
7.25	20	72000	19226719	13.153	13.153	36.817	-0.306
7.8	8	28800	9953117	6.824	6.824	43.176	-0.146
7.8	12	43200	16354212	11.213	11.213	38.787	-0.253
7.8	20	72000	21829113	14.967	14.967	53.633	-0.355
8.15	8	28800	3214059	2.303	2.303	47.797	-0.045
8.15	20	72000	7942591	5.446	5.446	44.554	-0.115
8.15	32	115200	12485289	8.56	8.56	41.44	-0.187
8.15	42	151200	16667941	11.428	11.428	38.572	-0.259
8.3	9	32400	50505471	4.808	4.808	46.535	-0.071
8.3	21	75600	6471263	4.76	4.76	45.563	-0.092
8.3	33	118800	11534260	4.589	4.589	42.092	-0.172
8.5	8	28800	4760484	3.264	3.264	46.736	-0.067
8.5	20	72000	10384732	7.12	7.12	42.88	-0.153
8.5	32	115200	22820196	15.647	15.647	34.353	-0.375
9.75	8.5	30600	3871610	2.546	2.546	47.454	-0.052
9.75	21	75600	8308284	5.969	5.969	44.304	-0.12
9.75	45	162000	18732412	12.844	12.844	37.156	-0.296
9.95	8	28800	2677350	1.835	1.835	48.165	-0.037
9.95	12	43200	8485957	5.818	5.818	44.182	-0.123
9.95	19	68400	14863251	10.191	10.191	39.8	-0.228
10.4	7	25200	2476276	1.697	1.697	48.309	-0.034
10.4	23	82800	9576098	6.566	6.566	43.434	-0.14
10.4	32	115200	19777693	13.561	13.561	36.439	-0.316
11.3	7	25200	1237349	0.848	0.848	49.152	-0.017
11.3	23	82800	4890005	3.352	3.352	46.648	-0.069
11.3	32	115200	9141689	6.268	6.268	43.372	-0.142
11.4	5	18000	1597488	1.095	1.095	48.905	-0.022
11.4	22	79200	5214260	3.575	3.575	46.425	-0.074
11.4	27.5	99000	7703566	5.282	5.282	44.718	-0.111
11.8	7	25200	1254628	0.86	0.86	49.14	-0.017
11.8	18.5	66600	6639396	4.552	4.552	45.44	-0.095
11.8	32	115200	12364281	8.478	8.478	41.52	-0.185
11.9	8	28800	8659321	5.937	5.937	44.063	-0.126
11.9	12	43200	12623651	8.655	8.655	41.345	-0.19
11.9	19	68400	19869121	13.623	13.623	36.377	-0.318
12.2	7	25200	1731129	1.186	1.186	48.814	-0.024
12.2	18.5	66600	4053929	2.779	2.779	47.221	-0.057
12.2	22	79200	5668579	3.886	3.886	46.114	-0.08

Table A-2 (continued)

12.2	27.5	99000	9534291	6.537	6.537	43.463	-0.14
12.46	7	25200	1623202	1.12	1.12	48.88	-0.022
12.46	19	68400	4646054	3.18	3.18	46.82	-0.065
12.46	24	86400	7072555	4.84	4.84	45.16	-0.101
12.46	30	108000	5151695	5.58	5.58	44.42	-0.118
12.6	7	25200	5403743	3.7	3.7	46.3	-0.076
12.6	19	68400	9571574	6.56	6.56	43.44	-0.14
12.6	24	86400	13236877	9.07	9.07	40.93	-0.2
12.6	30	108000	16914153	11.59	11.59	38.41	-0.263
12.7	7	25200	4954817	3.39	3.39	46.61	-0.07
12.7	23	82800	7208866	4.94	4.94	45.06	-0.104
12.7	29	104400	9448147	6.47	6.47	43.53	-0.138
12.7	48	172800	14496969	9.94	9.94	40.06	-0.221
12.85	7	25200	4349201	2.98	2.98	47.02	-0.061
12.85	23	82800	8562830	5.87	5.87	44.13	-0.125
12.85	29	104400	9993649	6.85	6.85	43.15	-0.147
12.85	48	172800	16760501	11.49	11.49	38.51	-0.261

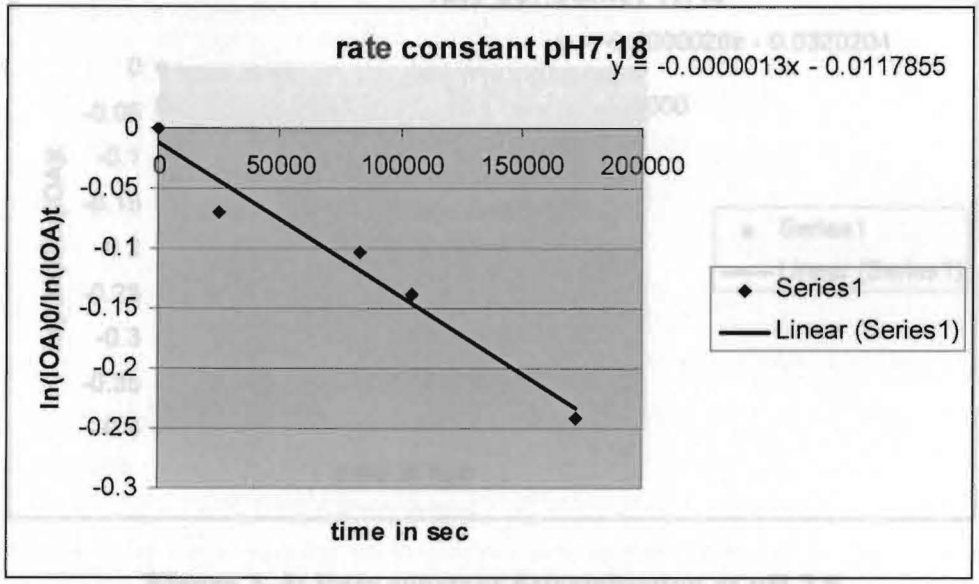


Figure A-2: Rate constant determination at pH 7.18

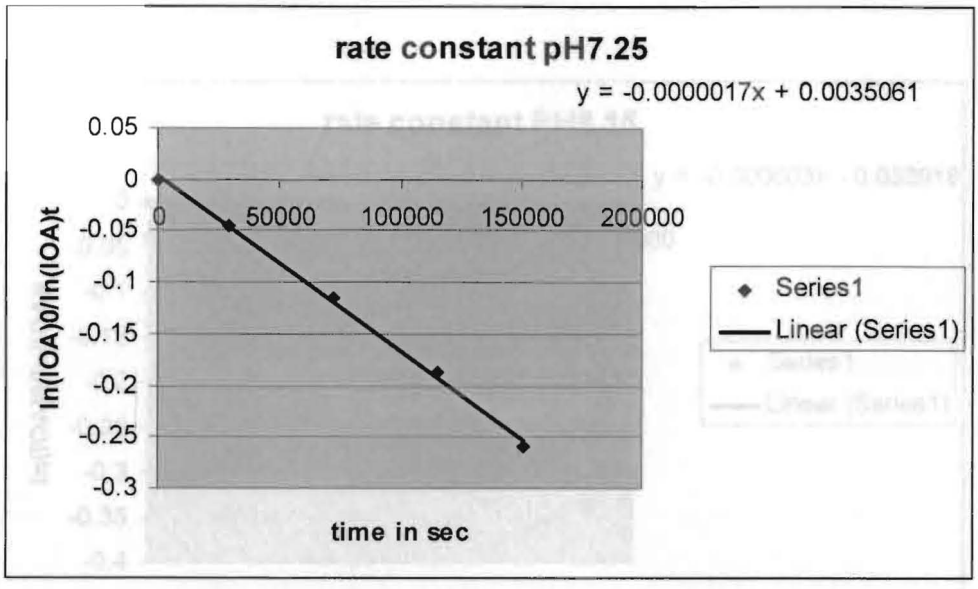


Figure A-3: Rate constant determination at pH 7.25

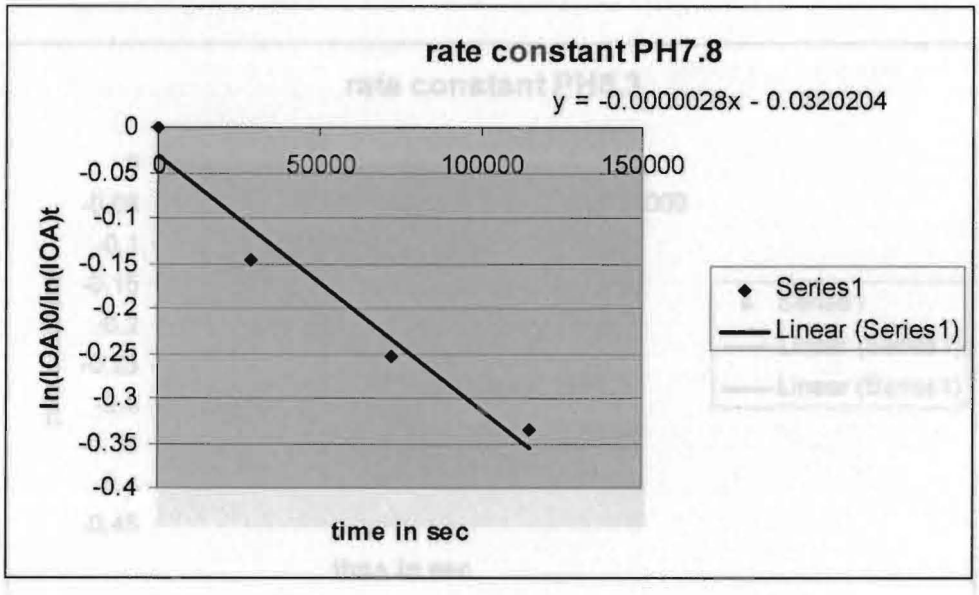


Figure A-4: Rate constant determination at pH 7.8

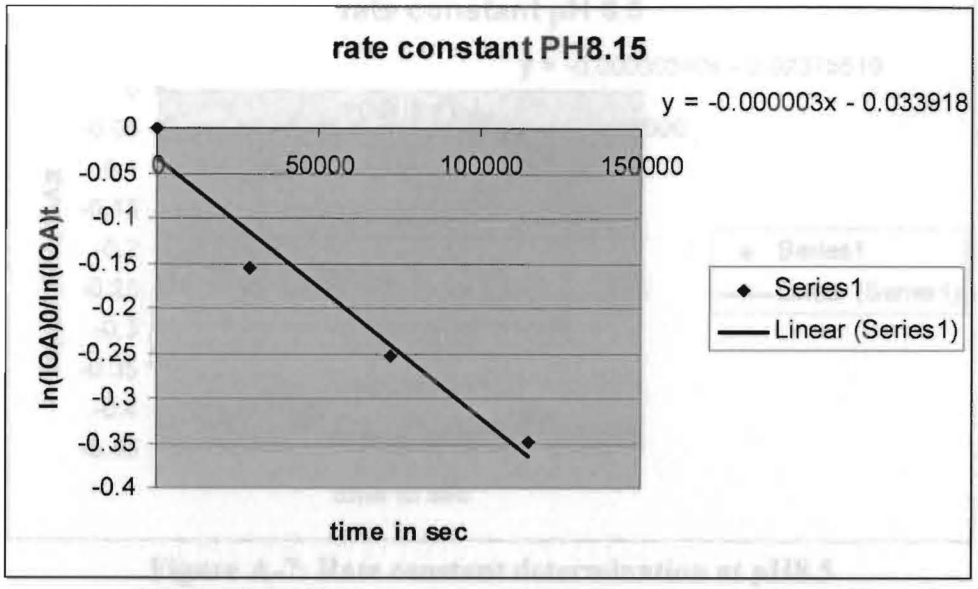


Figure A-5: Rate constant determination at pH 8.15

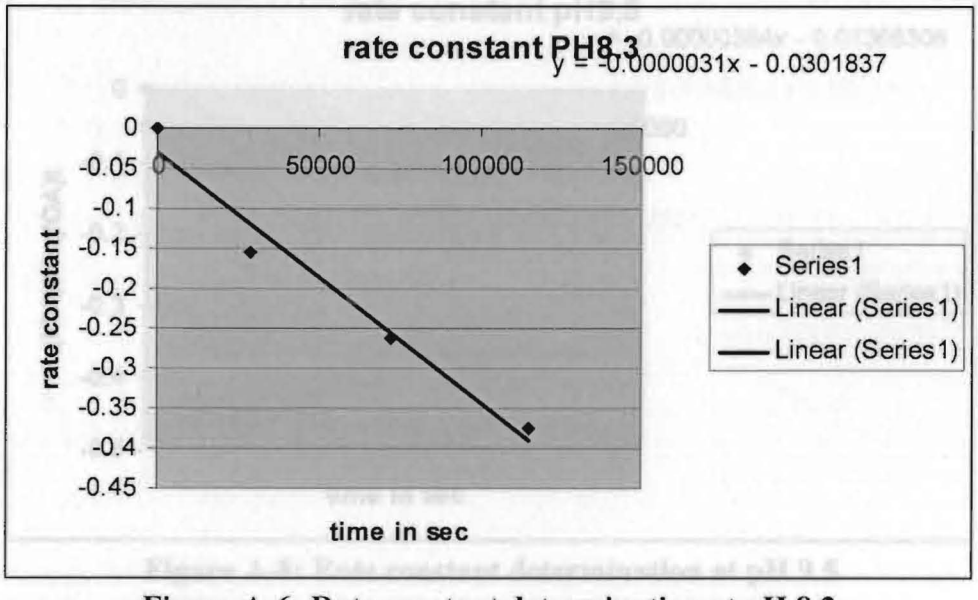


Figure A-6: Rate constant determination at pH 8.3

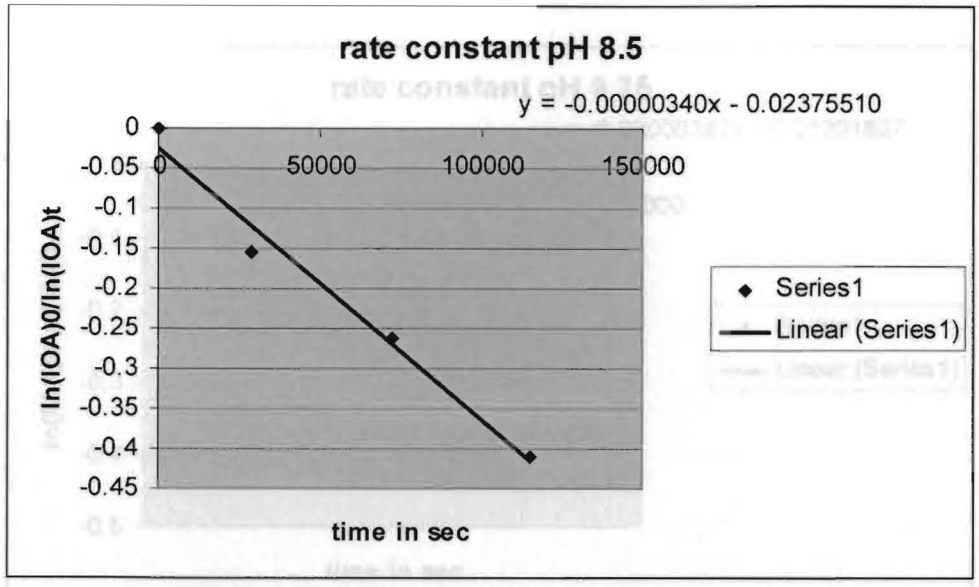


Figure A-7: Rate constant determination at pH8.5

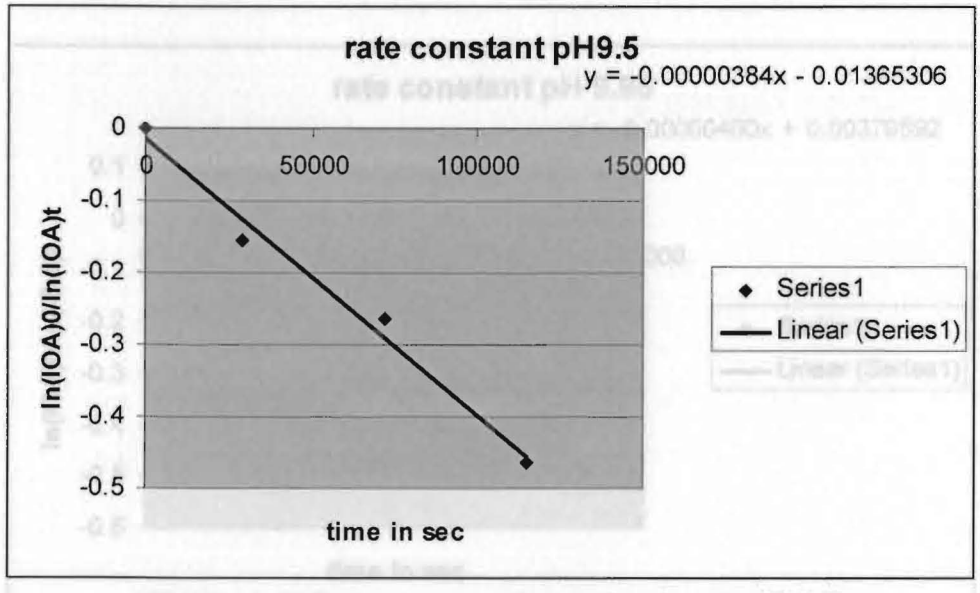


Figure A-8: Rate constant determination at pH 9.5

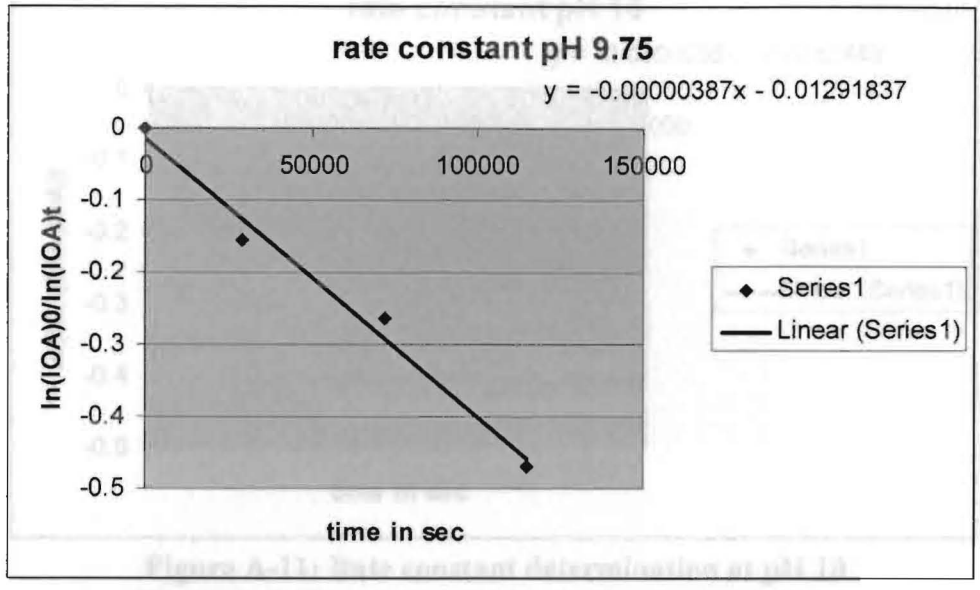


Figure A-9: Rate constant determination at pH 9.75

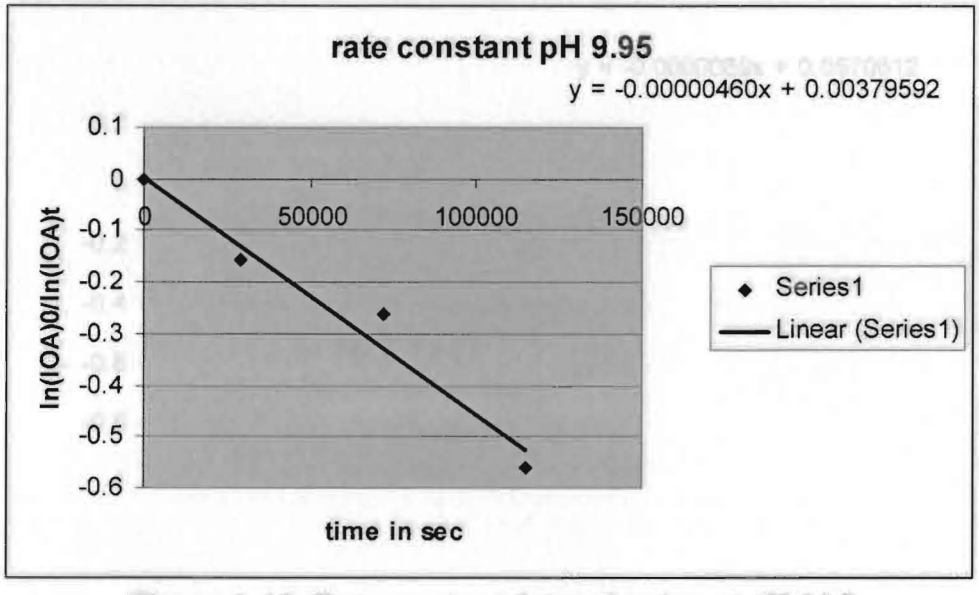


Figure A-10: Rate constant determination at pH 9.95

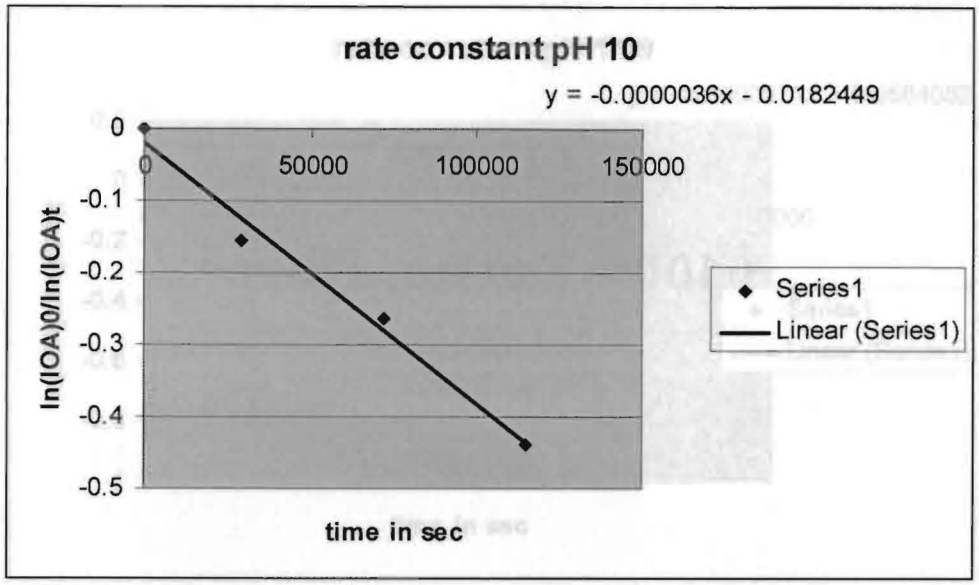


Figure A-11: Rate constant determination at pH 10

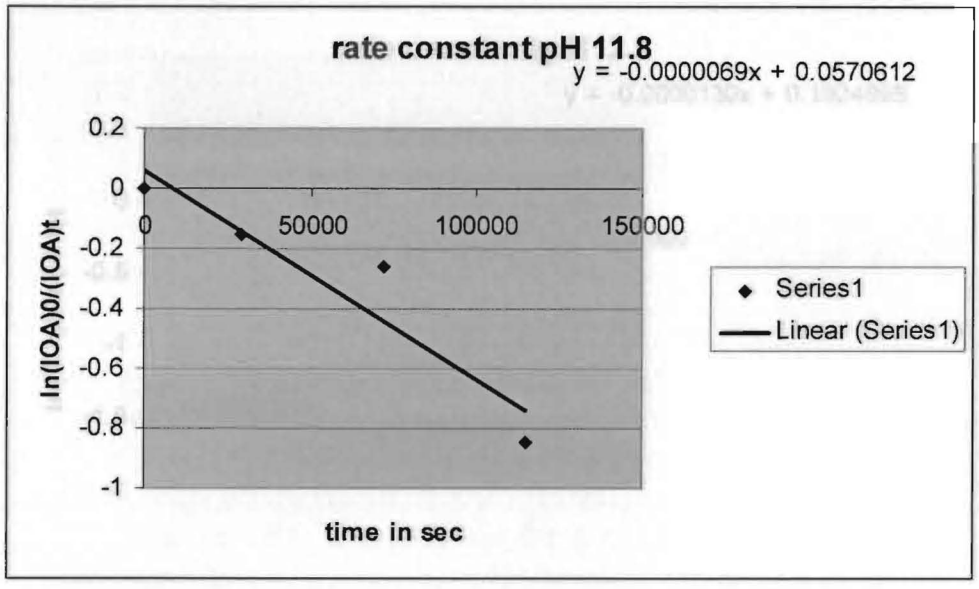


Figure A-12: Rate constant determination at pH 11.8

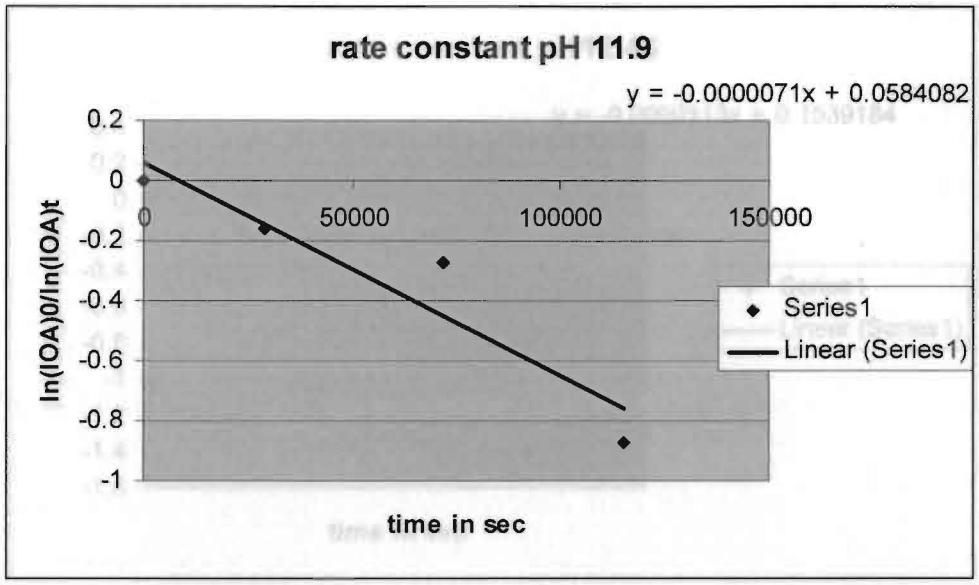


Figure A-13: Rate constant determination at pH 11.9

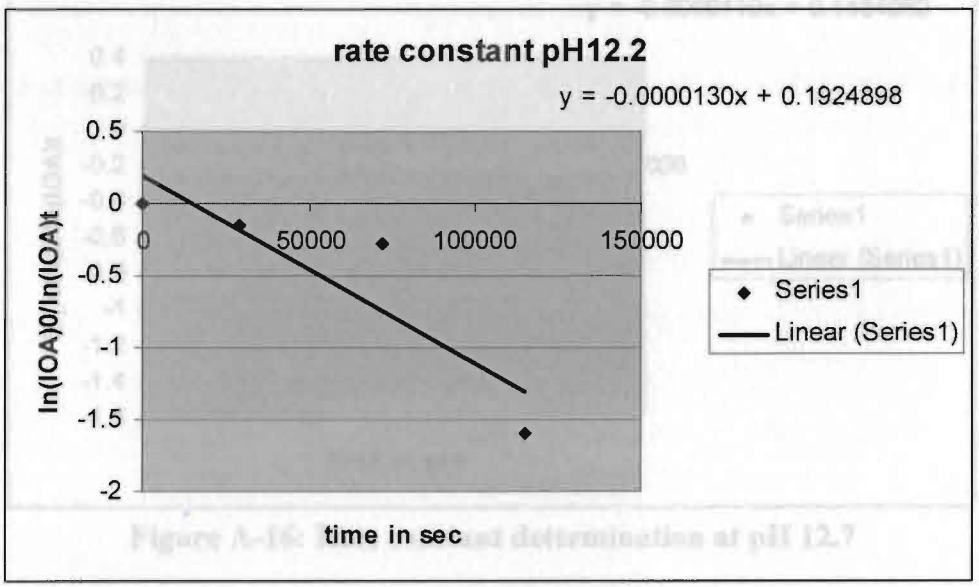


Figure A-14: Rate constant determination at pH 12.2

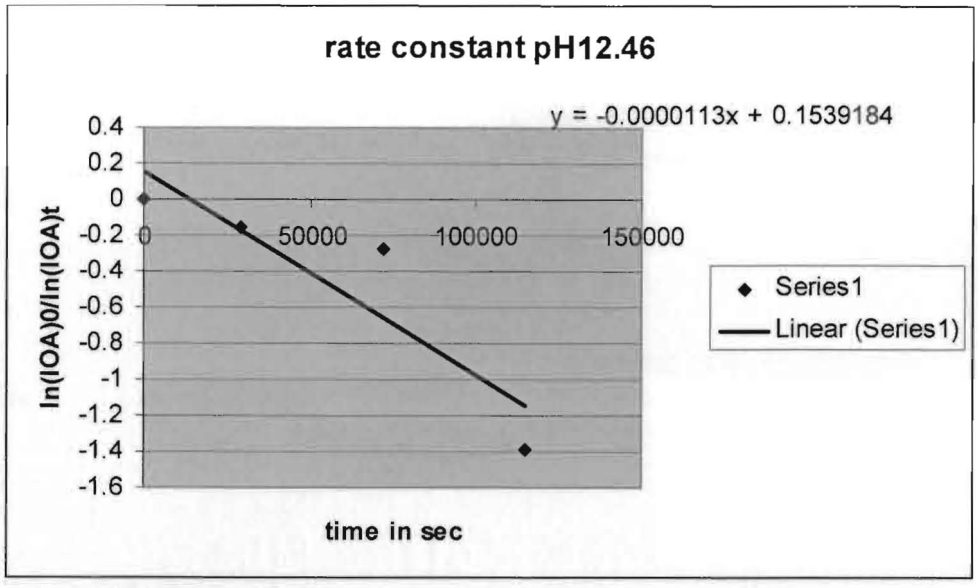


Figure A-15: Rate constant determination at pH 12.46

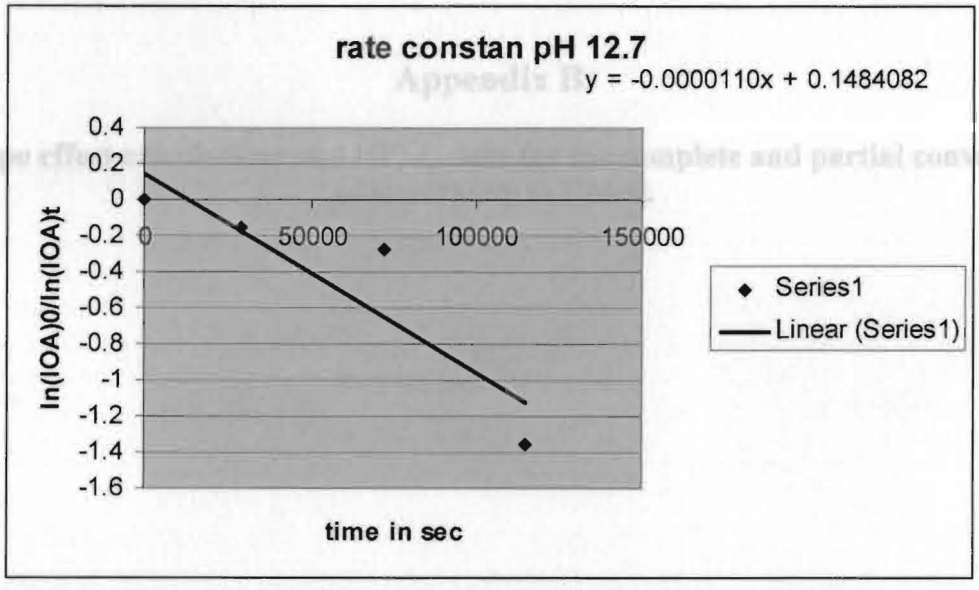


Figure A-16: Rate constant determination at pH 12.7

Appendix B:

Isotope effect calculations and HPLC data for the complete and partial conversion of Iso-orotate to Uracil.

CO_2 Isotope effect calculations:

Sample Information

Enzyme:	**Non-enzymatic IDCase**
Substrate:	**IOA Nat. abundance**
Concentration:	**10 mM**
Volume	**10 mL**
pH	**7.8**
Temp.	**100 C**
Buffer	**10 mM phosphate, no glycerol**
Reaction time	**60 min.**
mg Enzyme	

		1000 + δ
Fraction reaction (0.0 - 1.0)	0.24	
Delta value (partial rxn)	2.279	1002.279
Delta value (100% conversion)	21.855	1021.855
Isotope effect	1.0225	

User

Umakanth

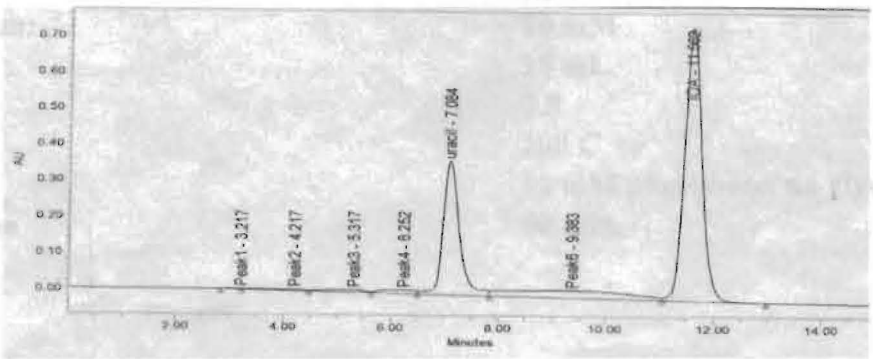


Figure B-1: HPLC data to determine partial reaction for IDCase isotope effect measurement, pH 7.8, trial 1.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	3.217	33454	0.11	2241	0.19
2	Peak2	4.217	242217	0.83	4123	0.35
3	Peak3	5.317	395149	1.36	7426	0.63
4	Peak4	6.252	459549	1.58	10749	0.91
5	uracil	7.084	7855479	26.95	370956	31.54
6	Peak6	9.383	3276022	11.24	20817	1.77
7	IOA	11.562	16891095	57.94	760015	64.61

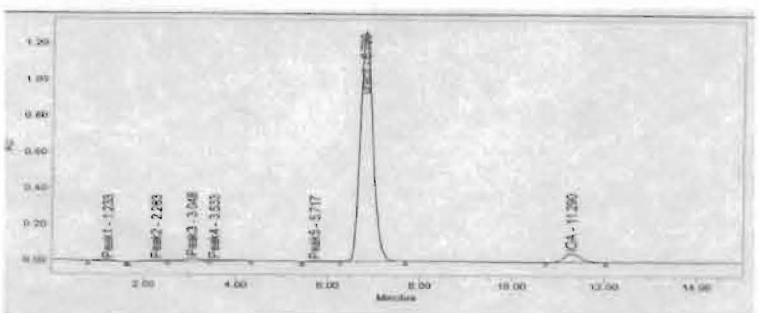


Figure B-2: HPLC data to determine complete reaction for IDCase isotope effect measurement, pH 7.8, trial 1.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	1.233	184407	0.69	6970	0.51
2	Peak2	2.283	271144	1.01	7628	0.56
3	Peak3	3.048	556850	2.07	19025	1.39
4	Peak4	3.533	151587	0.56	4468	0.33
5	Peak5	5.717	210988	0.78	5482	0.40
6	uracil	6.788	24333625	90.50	1271303	93.22
7	IOA	11.290	1179983	4.39	48950	3.59

CO_2 Isotope effect calculations:

Sample Information	
Enzyme:	**Non-enzymatic IDCase**
Substrate:	**IOA Nat. abundance**
Concentration:	**10 mM**
Volume	**10 mL**
pH	**7.8**
Temp.	**100 C**
Buffer	**10 mM phosphate, no glycerol**
Reaction time	**60 min.**
mg Enzyme	

Fraction reaction (0.0 - 1.0)	0.31
Delta value (partial rxn)	3.521
Delta value (100% conversion)	21.9
Isotope effect	1.0222

User

Umakanth

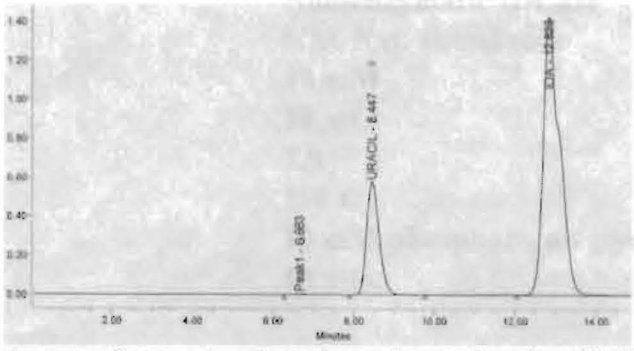


Figure B-3: HPLC data to determine fraction of reaction for IDCase isotope effect measurement, pH 7.8, trial 2.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	6.63	89362	0.17	1979	0.10
2	uracil	8.447	12594744	23.83	581736	28.90
3	IOA	12.823	40178860	76.01	1429325	71.00

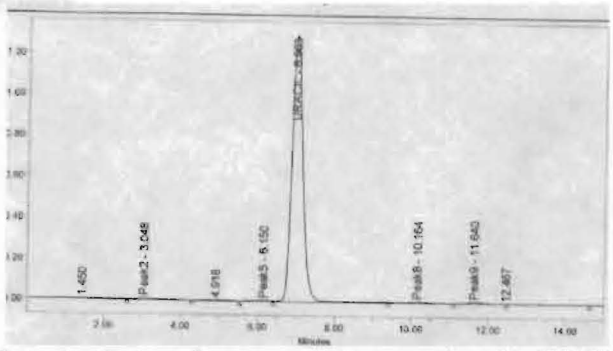


Figure B-4: HPLC data to determine complete reaction for IDCase isotope effect measurement, pH 7.8, trial 2.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	1.450	29876	0.11	2241	0.19
2	Peak2	3.048	242217	0.83	4123	0.35
3	Peak3	4.915	395149	1.36	7426	0.63
4	Peak5	6.150	459549	1.58	10749	0.91
5	uracil	6.933	7855479	95.95	370956	95.92
6	Peak8	10.154	32760221	1.24	20817	0.36
7	Peak9	11.645	16891095	0.94	760015	0.89

CO_2 Isotope effect calculations:

Sample Information

Enzyme:	**Non-enzymatic IDCase**
Substrate:	**IOA Nat. abundance**
Concentration:	**10 mM**
Volume	**10 mL**
pH	**7.8**
Temp.	**100 C**
Buffer	**10 mM phosphate, no glycerol**
Reaction time	**60 min.**
mg Enzyme	

Fraction reaction (0.0 - 1.0)	0.31	
		1000 + δ
Delta value (partial rxn)	3.521	1003.521
Delta value (100% conversion)	21.9	1021.9
Isotope effect	1.0222	

User

Umakanth

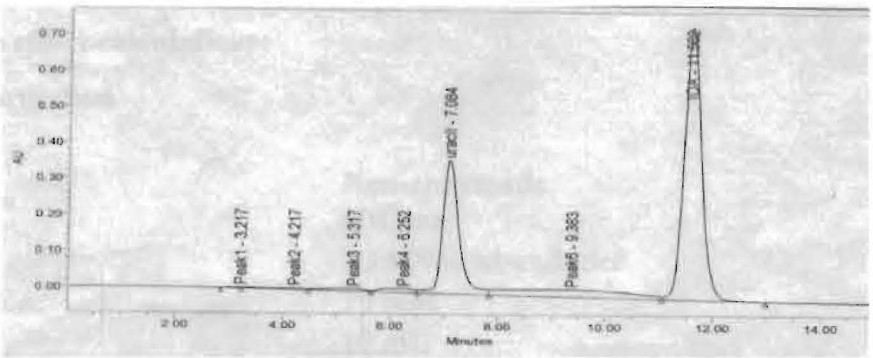


Figure B-5: HPLC data to determine fraction of reaction for IDCase isotope effect measurement, pH 7.8, trial 3.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	3.217	32454	0.11	2141	0.17
2	Peak2	4.217	232217	0.93	3923	0.59
3	Peak3	5.317	385149	1.26	7526	0.63
4	Peak4	6.252	448549	1.48	10749	1.01
5	uracil	7.084	7755479	25.95	360956	30.54
6	Peak6	9.383	2976022	12.24	19817	1.77
7	IOA	11.552	15891095	56.94	750015	63.61

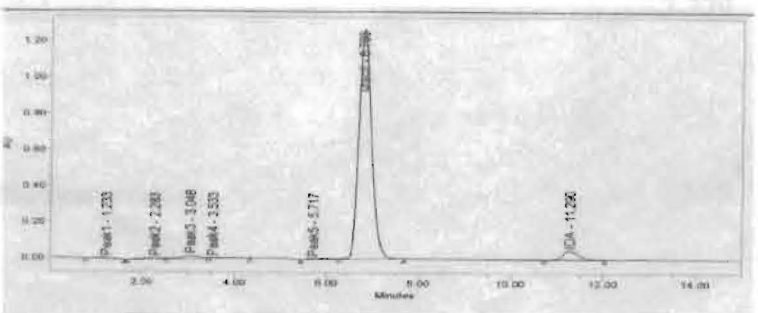


Figure B-6: HPLC data to determine complete reaction for IDCase isotope effect measurement, pH 7.8, trial 3.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	1.233	174417	0.49	6970	0.51
2	Peak2	2.283	203527	1.51	7628	0.56
3	Peak3	3.048	556850	1..97	19025	1.39
4	Peak4	3.533	151587	0.46	4468	0.33
5	Peak5	5.717	210988	0.78	5482	0.40
6	uracil	6.788	22333625	92.50	12171403	92.22
7	IOA	11.290	1278379	3.39	48950	3.59

CO_2 Isotope effect calculations:

Sample Information

Enzyme:	**Non-enzymatic IDCase**
Substrate:	**IOA Nat. abundance**
Concentration:	**10 mM**
Volume	**10 mL**
pH	**7.8**
Temp.	**100 C**
Buffer	**10 mM phosphate, no glycerol**
Reaction time	**60 min.**
mg Enzyme	

Fraction reaction (0.0 - 1.0)	0.27	
		$\underline{1000 + \delta}$
Delta value (partial rxn)	2.229	1002.229
Delta value (100% conversion)	20.99	1020.99
Isotope effect	1.0220	
User	Umakanth	

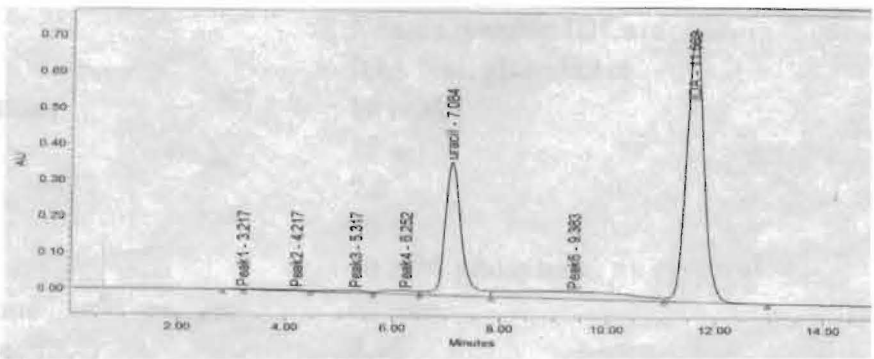


Figure B-7: HPLC data to determine fraction of reaction for IDCase isotope effect measurement, pH 7.8, trial 4.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	3.217	33454	0.11	2241	0.19
2	Peak2	4.217	242217	0.83	4123	0.35
3	Peak3	5.317	395149	1.36	7426	0.63
4	Peak4	6.252	459549	1.58	10749	0.91
5	uracil	7.084	7855479	26.95	370956	31.54
6	Peak6	9.383	3276022	11.24	20817	1.77
7	IOA	11.562	16891095	57.94	760015	64.61

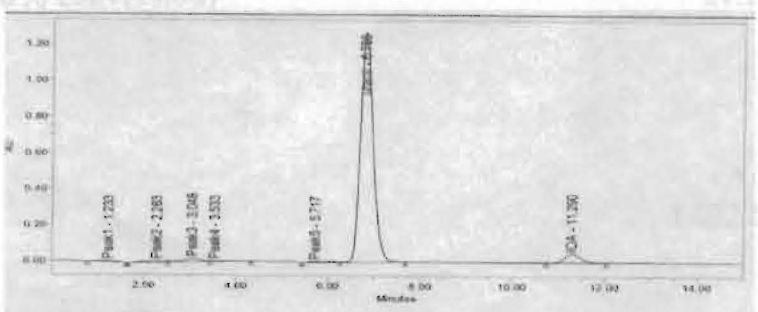


Figure B-8: HPLC data to determine complete reaction for IDCase isotope effect measurement, pH 7.8, trial 4

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	1.233	184407	0.69	6970	0.51
2	Peak2	2.283	271144	1.01	7628	0.56
3	Peak3	3.048	556850	2.07	19025	1.39
4	Peak4	3.533	151587	0.56	4468	0.33
5	Peak5	5.717	210988	0.78	5482	0.40
6	uracil	6.788	24333625	90.50	1271303	93.22
7	IOA	11.290	1179983	4.39	48950	3.59

CO_2 Isotope effect calculations:

Sample Information

Enzyme:	**Non-enzymatic IDCase**
Substrate:	**IOA Nat. abundance**
Concentration:	**10 mM**
Volume	**10 mL**
pH	**7.8**
Temp.	**30 C**
Buffer	**10 mM phosphate, no glycerol**
Reaction time	**60 min.**
mg Enzyme	**0.46**

		1000 + δ
Fraction reaction (0.0 - 1.0)	0.28	
Delta value (partial rxn)	2.301	1002.301
Delta value (100% conversion)	21.1	1021.1
Isotope effect	1.0222	

User

Umakanth

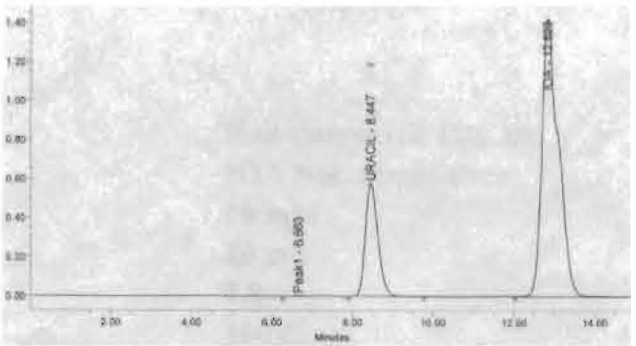


Figure B-9: HPLC data to determine fraction of reaction for IDCase isotope effect measurement, pH 7.8, trial 5.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	uracil	8.447	11493245	23.83	581736	31.90
2	IOA	12.823	41325678	76.01	1429325	69.52

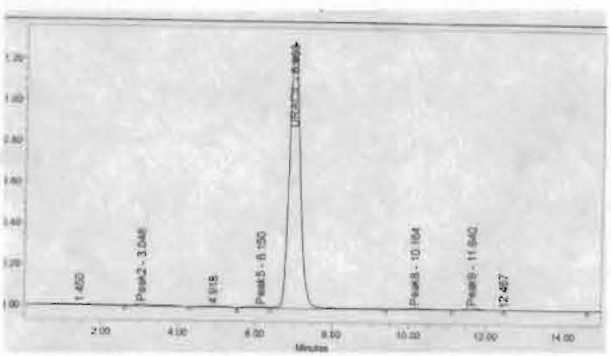


Figure B-10: HPLC data to determine complete reaction for IDCase isotope effect measurement, pH 7.8, trial 5.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	3.217	45274	0.11	2241	0.19
2	Peak2	4.217	262389	0.83	4123	0.35
3	Peak3	6.252	461235	1.58	10749	0.91
4	uracil	7.084	176543223	95.23	37095698	96.51
5	IOA	11.562	109876	1.72	654321	1.71

CO_2 Isotope effect calculations:

Sample Information

Enzyme:	**Non-enzymatic IDCase**
Substrate:	**IOA Nat. abundance**
Concentration:	**10 mM**
Volume	**10 mL**
pH	**7.8**
Temp.	**100 C**
Buffer	**10 mM phosphate, no glycerol**
Reaction time	**60 min.**
mg Enzyme	

Fraction reaction (0.0 - 1.0)	0.29	
		$\underline{1000 + \delta}$
Delta value (partial rxn)	2.399	1002.399
Delta value (100% conversion)	21.1	1021.1
Isotope effect	1.0222	

User

Umakanth

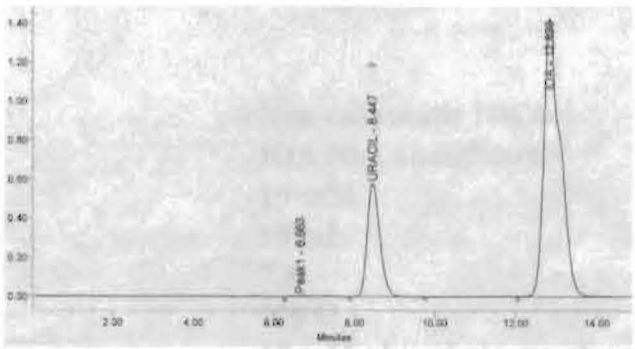


Figure B-11: HPLC data to determine fraction of reaction for IDCase isotope effect measurement, pH 7.8, trial 6.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	6.63	89362	0.17	1979	0.10
2	uracil	8.447	13894755	23.83	581736	26.76
3	IOA	12.823	42146660	76.01	1429325	73.00

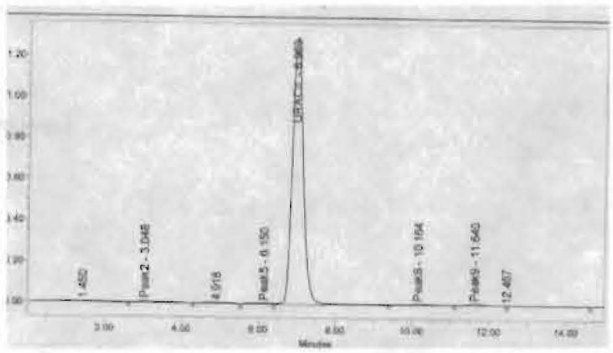


Figure B-12: HPLC data to determine complete reaction for IDCase isotope effect measurement, pH 7.8, trial 6.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	5.317	267543	1.36	7426	0.63
2	Peak2	6.252	339876	1.58	10749	0.91
3	uracil	7.084	781654365	98.65	5436778	98.24
4	Peak3	9.383	3276022	1.24	20817	1.77
5	IOA	11.562	168910	0.94	760015	0.98

CO_2 Isotope effect calculations:

Sample Information

Enzyme:	**Non-enzymatic IDCase**
Substrate:	**IOA Nat. abundance**
Concentration:	**10 mM**
Volume	**10 mL**
pH	**7.8**
Temp.	**100 C**
Buffer	**10 mM phosphate, no glycerol**
Reaction time	**60 min.**
mg Enzyme	

Fraction reaction (0.0 - 1.0)	0.32	**$\frac{1000 + \delta}{}$**
Delta value (partial rxn)	3.51	1003.51
Delta value (100% conversion)	21.7	1021.7
Isotope effect	1.0221	

User

Umakanth

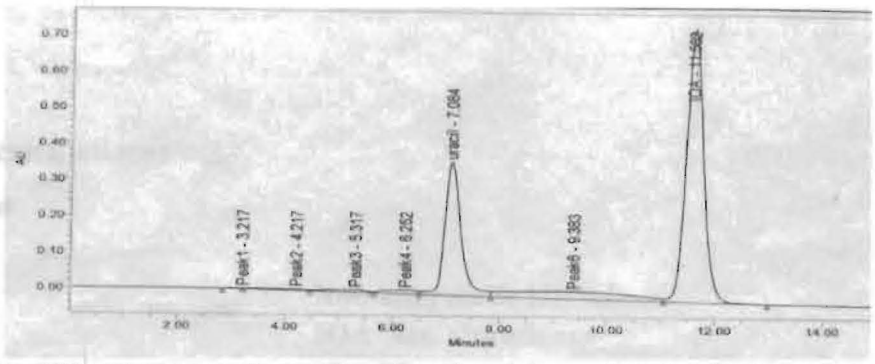


Figure B-13: HPLC data to determine fraction of reaction for IDCase isotope effect measurement, pH 7.8, trial 7.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
5	uracil	7.084	7655479	24.95	370956	31.54
7	IOA	11.562	17321098	59.94	760015	66.82

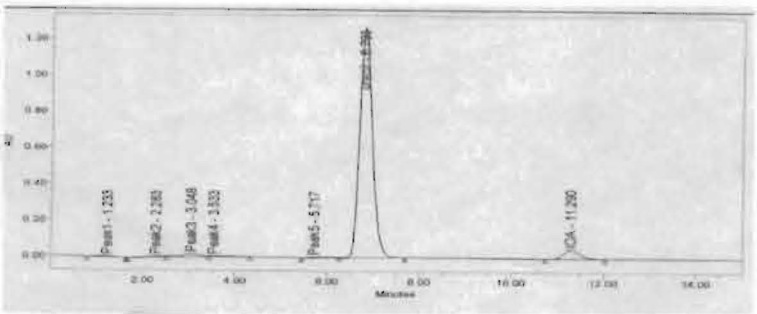


Figure B-14: HPLC data to determine complete reaction for IDCase isotope effect measurement, pH 7.8, trial 7.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	1.233	184407	0.69	6970	0.51
2	Peak2	2.283	271144	1.01	7628	0.56
3	Peak3	5.717	210988	0.78	5482	0.40
4	uracil	6.788	24333625	90.50	1379303	95.22
5	IOA	11.290	1179983	4.39	234567	1.59

CO_2 Isotope effect calculations:

Sample Information

Enzyme:	**Non-enzymatic IDCase**
Substrate:	**IOA Nat. abundance**
Concentration:	**10 mM**
Volume	**10 mL**
pH	**7.8**
Temp.	**100 C**
Buffer	**10 mM phosphate, no glycerol**
Reaction time	**60 min.**
mg Enzyme	**0.46**

Fraction reaction (0.0 - 1.0)	0.22	**$\frac{1000 + \delta}{}$**
Delta value (partial rxn)	2.8	1002.8
Delta value (100% conversion)	23	1023
Isotope effect	1.0229	

User

Umakanth

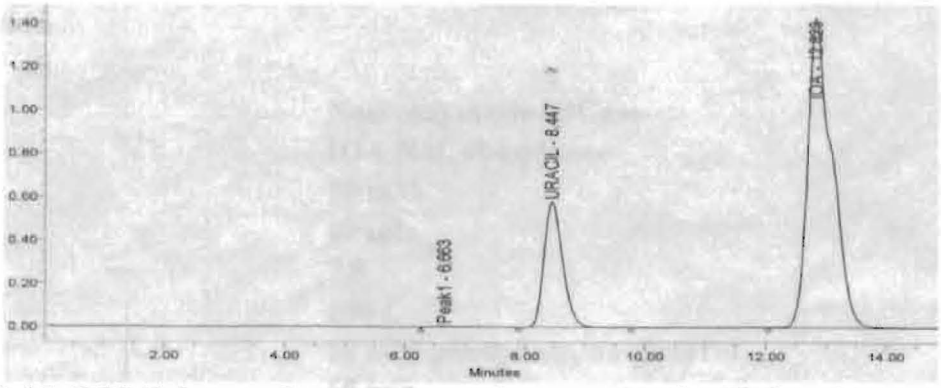


Figure B-15: HPLC data to determine complete reaction for IDCase isotope effect measurement, pH 7.8, trial 8.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	uracil	8.447	13494699	23.83	581736	29.90
2	IOA	12.823	41079850	76.01	1429325	72.00

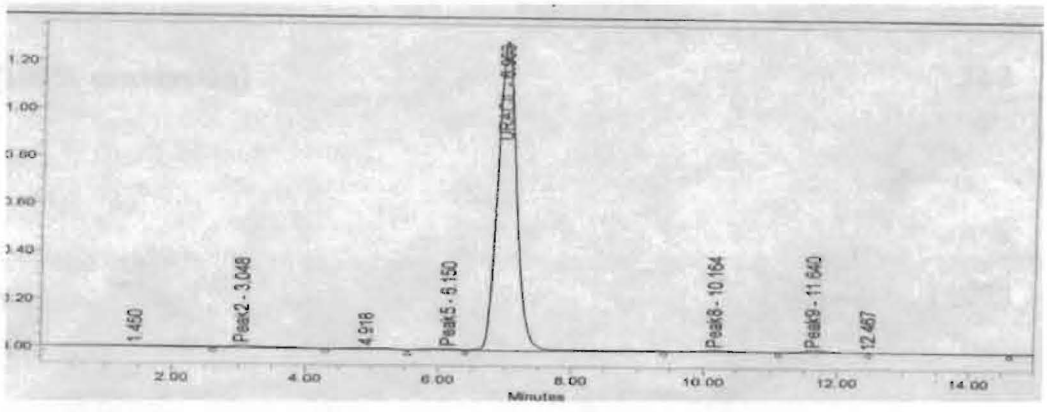


Figure B-16: HPLC data to determine complete reaction for IDCase isotope effect measurement, pH 7.8, trial 8.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	1.450	32979	0.11	2241	0.19
2	Peak2	3.048	282916	0.83	4123	0.35
3	Peak3	4.918	406754	1.36	7426	0.63
4	Peak4	6.150	456778	1.58	10749	0.91
5	uracil	6.933	8055479287	26.95	9709561	96.1
6	Peak5	10.164	287653	11.24	20817	0.98
7	IOA	11.640	189102	57.94	760015	0.23

CO_2 Isotope effect calculations:

Sample Information

Enzyme:	**Non-enzymatic IDCase**
Substrate:	**IOA Nat. abundance**
Concentration:	**10 mM**
Volume	**10 mL**
pH	**7.8**
Temp.	**100 C**
Buffer	**10 mM phosphate, no glycerol**
Reaction time	**60 min.**
mg Enzyme	

Fraction reaction (0.0 - 1.0)	0.33	
		1000 + δ
Delta value (partial rxn)	3.6	1003.6
Delta value (100% conversion)	22.2	1022.2
Isotope effect	1.0228	

User

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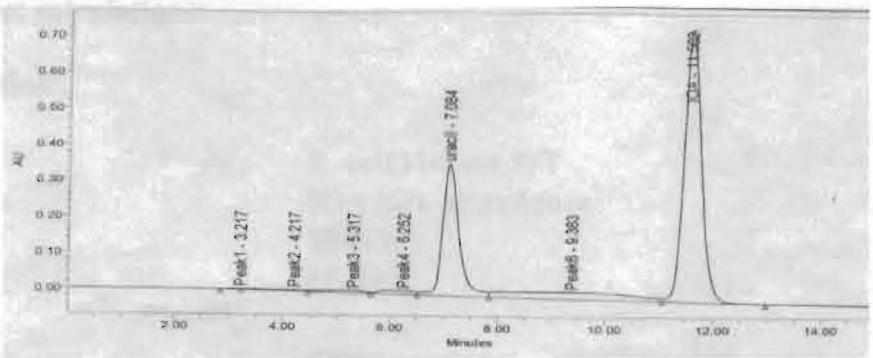

Figure B-17: HPLC data to determine fraction of reaction for IDCase isotope effect measurement, pH 7.8, trial 9.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	3.217	33454	0.11	2241	0.19
2	Peak2	4.217	242217	0.83	4123	0.35
3	Peak3	5.317	395149	1.36	7426	0.63
4	Peak4	6.252	459549	1.58	10749	0.91
5	uracil	7.084	7855479	26.95	370956	31.54
6	Peak6	9.383	3276022	11.24	20817	1.77
7	IOA	11.562	16891095	57.94	760015	64.61

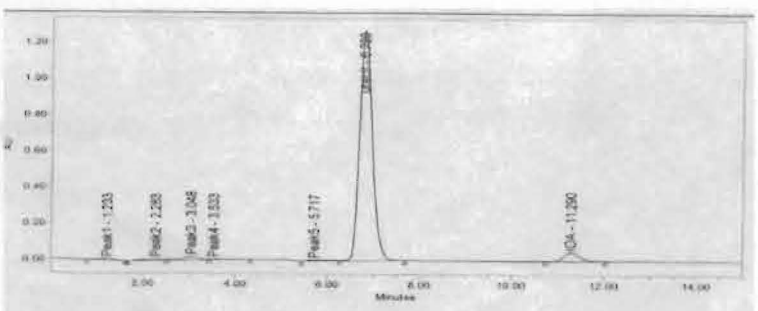

Figure B-18: HPLC data to determine complete reaction for IDCase isotope effect measurement, pH 7.8, trial 9.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	1.233	184407	0.69	5970	0.41
2	Peak2	2.283	271144	1.01	4628	0.56
3	Peak3	3.048	556850	2.07	16543	1.39
4	Peak4	3.533	151587	0.56	4468	0.33
5	Peak5	5.717	210988	0.78	5482	0.40
6	uracil	6.788	24333625	90.50	1421568	97.22
7	IOA	11.290	1179983	4.39	24650	0.99

CO_2 Isotope effect calculations:

Sample Information

Enzyme:	**E. coli IDCase WT**
Substrate:	**IOA Nat. abundance**
Concentration:	**10 mM**
Volume	**10 mL**
pH	**7.8**
Temp.	**30 C**
Buffer	**10 mM phosphate, no glycerol**
Reaction time	**60 min.**
mg Enzyme	**0.46**

Fraction reaction (0.0 – 1.0)	0.29	
		$\frac{1000 + \delta}{}$
Delta value (partial rxn)	3.6	1003.6
Delta value (100% conversion)	22.2	1022.2
Isotope effect	1.0221	

User

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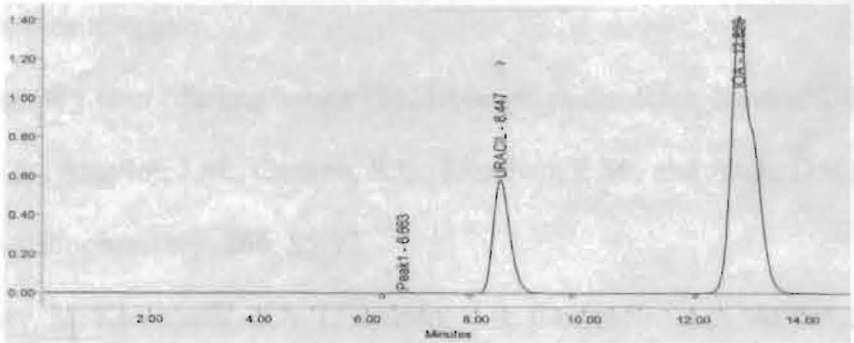

Figure B-19: HPLC data to determine fraction of reaction for IDCase isotope effect measurement, pH 7.8, trial 10.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	6.63	89362	0.17	1979	0.10
2	uracil	8.447	12594744	23.83	581736	28.90
3	IOA	12.823	40178860	76.01	1429325	71.00

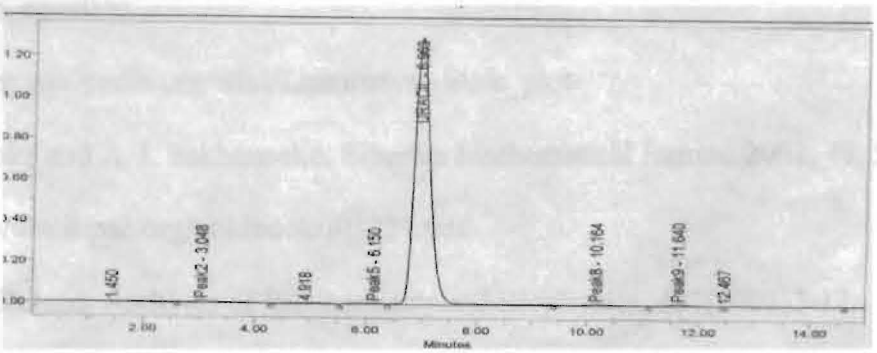

Figure B-20: HPLC data to determine complete reaction for IDCase isotope effect measurement, pH 7.8, trial 10.

	Peak name	RT (min)	Area (μVsec)	%Area	Height (μV)	% Height
1	Peak1	2.167	33454	0.11	2241	0.19
2	Peak2	3.048	242217	0.83	4123	0.35
3	Peak5	6.150	395149	1.36	7426	0.63
4	Peak4	6.252	459549	1.58	10749	0.91
5	uracil	6.963	7855479	26.95	380956	31.54
6	Peak9	11.640	3276022	11.24	20817	1.77
7	Peak10	12.467	17011095	57.94	760015	68.31

References:

1) www.visionlearning.com

2) www.pharmacy.umn.edu/img/assets/12621/biosyn_nucleotides_handout1-04a.doc

3) Smiley, J.A., Angelot, J.M., Cannon, R.C., Marshall, E.M., and Asch, D.K (1999). *Analytical Biochemistry*, **266**, 85-92.

4) J.A. Smiley, M. Kundracik, D.A. Landfried, V.R. Barnes Sr., A.A. Axhemi, *Biochimica et Biophysica Acta* 1723 (2005) 256– 264.

5) V.R. Barnes Sr. Master's Thesis. Youngstown State University.

6) M.L. Shuler and F. Kargi Bioprocess Engineering Basic Concepts

7) D.Voet and J.G.Voet. Biochemistry: 2nd Ed. (1995)

8) www.chem.qmul.ac.uk/iubmb/kinetics/ek4t6.html#p42

9) Matthews, J. N. S; Allcock, G. C. Statist. Med. 2004; 23:477–491

10) www.en.wikipedia.org/wiki/Enzyme_kinetics#Linear_plots_of_the_Michaelis-Menten_equation

11) www.en.wikipedia.org/wiki/Lineweaver-Burk_plot

12) Yu. Linke and A. I. Sakhanenko, Siberian Mathematical Journal.2001, 42, 517–536.

13) http://www.iupac.org/goldbook/I03327.pdf

14) W.W.Cleland. Archives of Biochemistry and Biophysics, 433(2005)2-12.

15) M.I. Schimerlik, J.E. Rife, W.W. Cleland, Biochemistry 14 (1975) 5347–5354.

16) W.W. Cleland, M.H. O_Leary, D.B. Northrop (Eds.), Isotope Effects on Enzyme-catalyzed Reactions, University Park Press, Baltimore, MD, 1977, pp. 271–283

17) D.B. Northrop, Biochemistry 14 (1975) 2644–2651.

18) M.A. Rishavy, W.W. Cleland, Biochemistry 39 (2000) 7546–7551