Elucidating the mechanism of action of Orotidine Monophosphate Decarboxylase (ODCase) using Isotope labeling ,enzyme kinetics and 6-CN-UMP inhibitor

by

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ABSTRACT

Orotidine 5-Monophosphatedecarboxylase (ODCase) is involved in the de novo biosynthesis of Uridine 5-Monophosohate (UMP) via a decarboxylation reaction using Orotidine 5-monophosphate as the substrate. UMP is used in the synthesis of pyrimidines which are important components of the nucleotides. ODCase is one of the most proficient enzymes ,however little is known about its mechanism of action. We are concerned with ascertaining the validity of the proposed
ODCase mechanism of action via a zwitterionic intermediate followed by subsequent decarboxylation at C6. By synthesizing a double labeled substrate(OMP) with the ^{13}C labeled carbon at the carbonyl carbon at position 6 (the key position) and ^{18}O labeled at position 2 (indicator position) , the reaction is run to partial completion using the *E . coli* system. A separate experiment is run for the naturally occurring OMP. By carrying out isotope effect calculations we can ascertain whether O2 is involved in the ODCase mechanism of action or not[1].

According to *Masahiro Fujihashi*[2] when ODCase from M. thermoautotrophicium was incubated with 6-CN-UMP showed diminished activity due to the formation of BMP a potent inhibitor. We are concerned in finding out if the same happens with ODCase from *E. coli* and whether the oxygen in
BMP comes from water or dissolved oxygen

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LIST OF SYMBOLS AND ABREVEATIONS

ODCase	Orotidine 5'-monophosphate decarboxylase
OMP	Uridine 5'-monophosphate
BMP	1-(5'-phospho-β-D-ribofuranosyl) babituric acid
IPTG	Isopropyl-β-Dthiogalactopyranoside

Chapter 1: Introduction

Decarboxylases

Decarboxylases are enzymes that catalyze the splitting of the carboxyl group from a carboxylic acid to release carbon dioxide.

$$R{-}C(=O){-}OH \longrightarrow R{-}H + CO_2$$

Figure 1-1: General reaction scheme for a decarboxylation.

Common biosynthetic decarboxylations of amino acids to amines are: tryptophan to tryptamine, phenylalanine to phenylethylamine and tyrosine to tyramine. Chemical decarboxylations reactions often require extensive heating in high-boiling solvents. Copper salts are often added as catalysts.

Orotidine5'-monophosphate decarboxylase(ODCase) is involved in the *de novo* biosynthesis of uridine 5-monophosphate (UMP) via a decarboxylation reaction. This reaction is an essential step in the pyrimidine biosynthetic pathway and has intrigued enzymologists [3].

OMP $\xrightarrow{H^+,\ -CO_2}$ UMP

Figure 1-2: The reaction of ODCase.

ODCase is one of the most proficient enzymes with k_{cat}/k_{uncat} of 10^{17} (18 ms for a reaction that takes 78 million years in an uncatalyzed reaction) [2]. The enzyme is unique in that it functions without any metals or co-factors yet the substrate is devoid of an effective repository for the negative charge that is generated at C-6 when CO_2 is generated. Atomic absorption and X-ray absorption spectroscopic studies did not detect the presence of any transition metals [4]. Figure 1-3 shows the rate of enhancement of different enzymes; ODCase has one of the largest rates of rate enhancement indicated by the length of the red line [5].

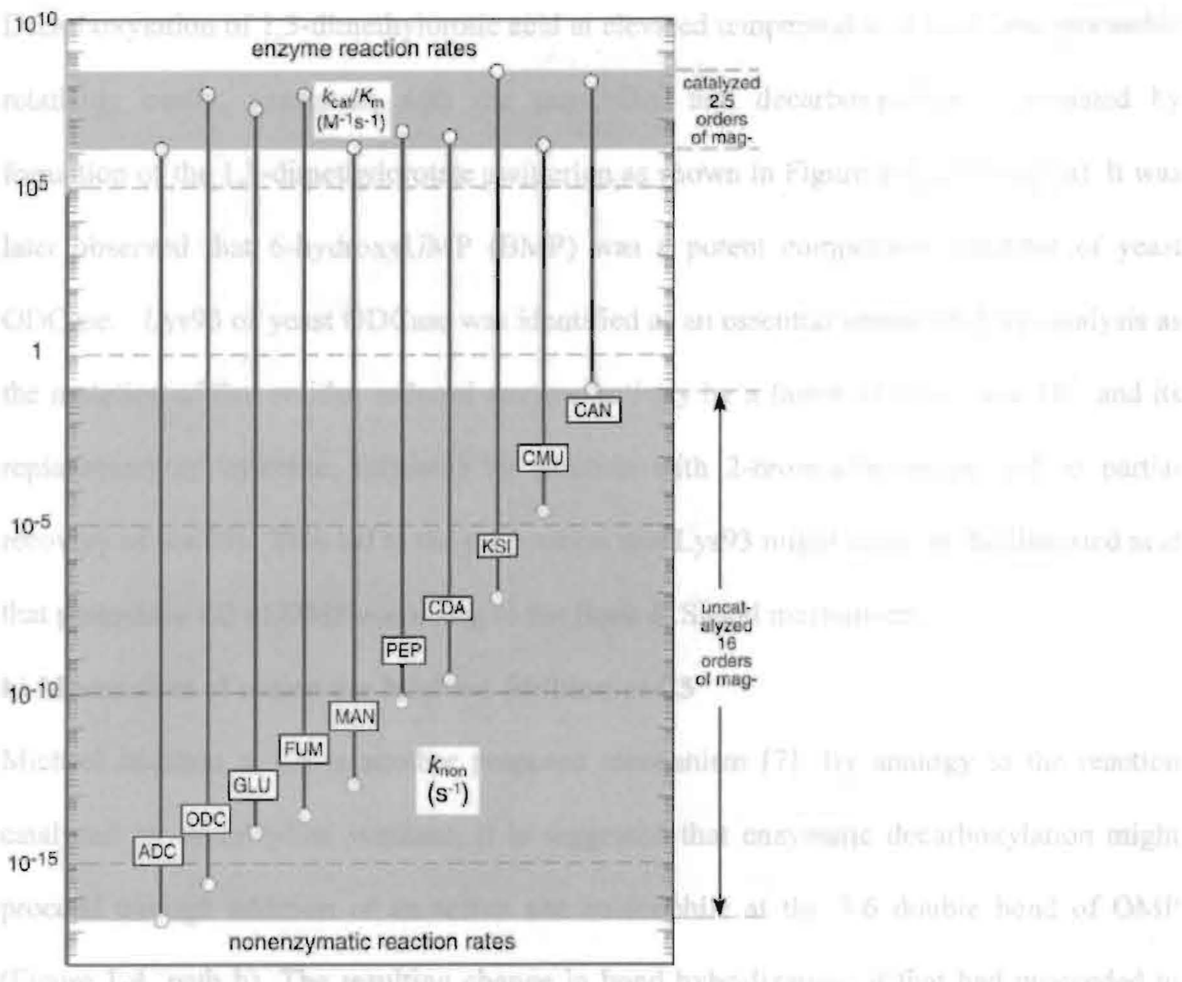


Figure 1-3 - B. G. Miller and R. Wolfenden, Reference [3]. ADC - Arginine decarboxylase; ODC - ODCase; PEP - Carboxypeptidase; CAN - carbonic anhydrase.

Mechanism of action of ODcase

Several mechanisms have been proposed for the ODcase decarboxylation reactions as follows:

a) Mechanism via a zwitterion formation

According to Beak and Siegel [6], 2-methylation of 1-methylorotate acid led to an increase of more than 10^8-fold in the rate of spontaneous decarboxylation. It is proposed that an acidic group on the enzyme might perform a similar function by protonating O2 of the substrate, to generate a zwitterionic form of OMP with a proton attached to N3. Decarboxylation of 1,3-dimethylorotic acid at elevated temperatures in sulfolane proceeded relatively easily, consistent with the possibility that decarboxylation is initiated by formation of the 1,3-dimethylorotate zwitterion as shown in Figure 1-4, pathway (a). It was later observed that 6-hydroxyUMP (BMP) was a potent competitive inhibitor of yeast ODCase. Lys93 of yeast ODCase was identified as an essential amino acid for catalysis as the mutation of that residue reduced enzyme activity by a factor of more than 10^7; and its replacement by cysteine, followed by reaction with 2-bromoethylamine, led to partial recovery of activity. This led to the proposition that Lys93 might serve as the Brønsted acid that protonates O2 of OMP according to the Beak & Siegel mechanism.

b) Mechanism of action via Michael addition at C5

Michael addition at C5 is another proposed mechanism [7]. By analogy to the reaction catalyzed by thymidylate synthase, it is suggested that enzymatic decarboxylation might proceed through addition of an active site nucleophile at the 5-6 double bond of OMP (Figure 1-4, path b). The resulting change in bond hybridization, if that had proceeded to any considerable extent during the rate-determining step, would be expected to lead to a

secondary kinetic isotope effect on k_{cat}/K_m if deuterium were substituted at C5, and to a change in electron density at C5 if the potential transition state analog 6-hydroxyUMP

Figure 1-4: Five mechanisms for ODCase catalysis suggested in various publications. From B. G. Miller and R. Wolfenden, Reference [3].

were similarly bound. When these possibilities were tested (the latter effect by ^{13}C NMR, using 6-hydroxyUMP labeled with ^{13}C at the 5-position), neither prediction was fulfilled.

c) Proposed carbene mechanism

Lee and Houk [8] proposed the carbene mechanism at carbon 6 (Figure 1-4, path c). Gas-phase proton affinities, calculated for orotate by Lee and Houk, suggest that O4 rather than O2 is the most likely site of protonation of OMP in the vapor phase, and simulations of the reaction in a nonpolar environment suggest that the spontaneous decarboxylation of orotate may proceed by a mechanism that involves protonation at O4, followed by formation of a neutral carbene at C6. However, no acidic residue appears to be present near O4 of a bound substrate analog, a bound transition state analog, or the reaction product, reducing the likelihood of a simple carbene-based mechanism

d) Mechanism via direct protonation of C6 by lysine-93

Based on the crystal structure of the *Bacillus subtilis* enzyme in complex with product UMP, Begley and associates proposed electrophilic substitution reaction (Figure 1-4, path d). This mechanism postulates that Lys93 protonates the fragmentation of C-C bond once that bond becomes sufficiently basic. Recently studies indicate that the acidity of C-6 proton of 1,3 dimethyluracil is estimated to be $pK_a \approx 34$, demonstrating the difficulty with which the C-6 carbanion is formed.

An unusual side reaction of ODCase involving formation of BMP from CN-UMP

Fujihashi *et al.* [9] proposed that contrary to the natural biochemical transformation of OMP to UMP, which is thought to progress through a nucleophilic intermediate, the transformation of OMP to UMP possibly may include an electrophilic center. The researchers used 6-CN-UMP as the substrate which mimics the electron rich carbonyl group; this was converted to BMP leading them to propose a mechanism via an electrophilic centre. Mass spectral analysis of ODCase incubated with 6-CN-UMP showed conversion to BMP. X-ray electron density maps indicated the electropositive nature of C6 and X-ray crystal structures showed BMP bound to the enzyme.

The figure below shows Mass spectral analyses of ODCase with 6-CN-UMP (for 0 h and 7 day incubations at room temperature shown in panels A and B, respectively). A shift in the peaks of ODCase from 27 344.6 to 27 694.0 (peaks A and D, panel A) is due to the formation of the ODCase complex and with 6-CN-UMP (new molecular weight indicates the formation of the complex between ODCase and BMP.

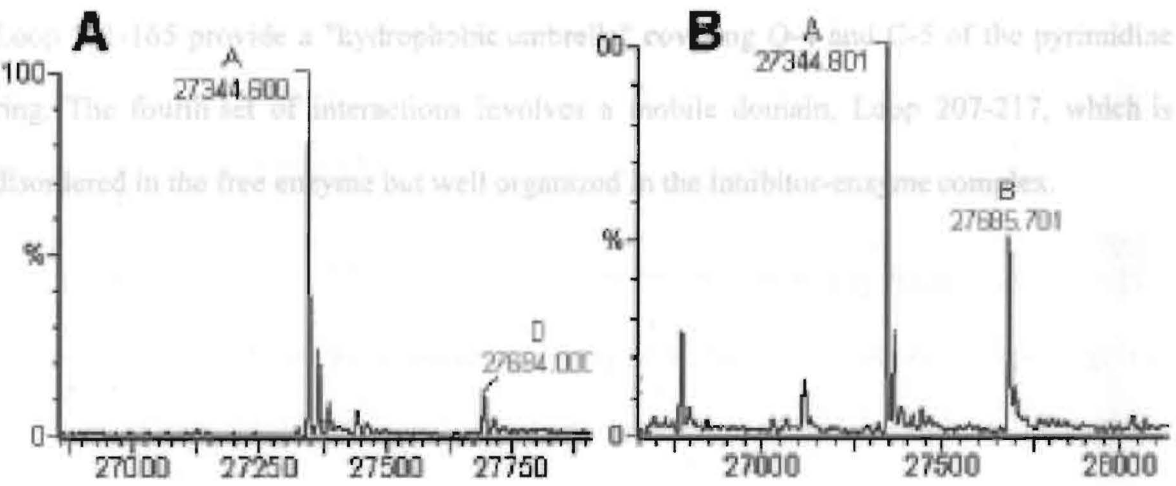


Figure 1-5: Mass spectrometric analysis of ODCase/6-CN-UMP mixtures. From Fujihashi *et al.*, Reference [9].

Proposed structure of substrate bound to the ODCase active site

Miller *et al.* [10] used BMP as an inhibitor to demonstrate the binding of OMP to the active site of ODCase. H-bonds between BMP and ODCase can be divided into four groups based on their position and function in the free and inhibited enzymes. One group of H-bonds between BMP and enzyme involves a scaffold of residues whose positions are little affected by ligand binding, changing their $C\alpha$ positions by 0.6 Å, on average. These scaffold H-bonds are formed from Gly-234(NH) to the phosphoryl group, from Asp-37 to the 3'-OH group, from Lys-93 to O-6, and from Arg-235 to the phosphoryl group. Arg-235 was included in this group based on function because its $C\alpha$ rms deviation movement is 0.96 Å. In a second group, residues from the opposite subunit extend across the dimer interface into the binding pocket, forming H-bonds from Asp-96and Thr-100 to the 2'-OH group of BMP. These contacts are made possible by a moderate repositioning of Loop 95-103 as BMP is bound. Third, Loop 151-165 shifts position, moving toward the bound ligand. The peptide amide of Ser-154 contacts O-4 of BMP. The remaining residues of Loop 151-165 provide a "hydrophobic umbrella" covering O-4 and C-5 of the pyrimidine ring. The fourth set of interactions involves a mobile domain, Loop 207-217, which is disordered in the free enzyme but well organized in the inhibitor-enzyme complex.

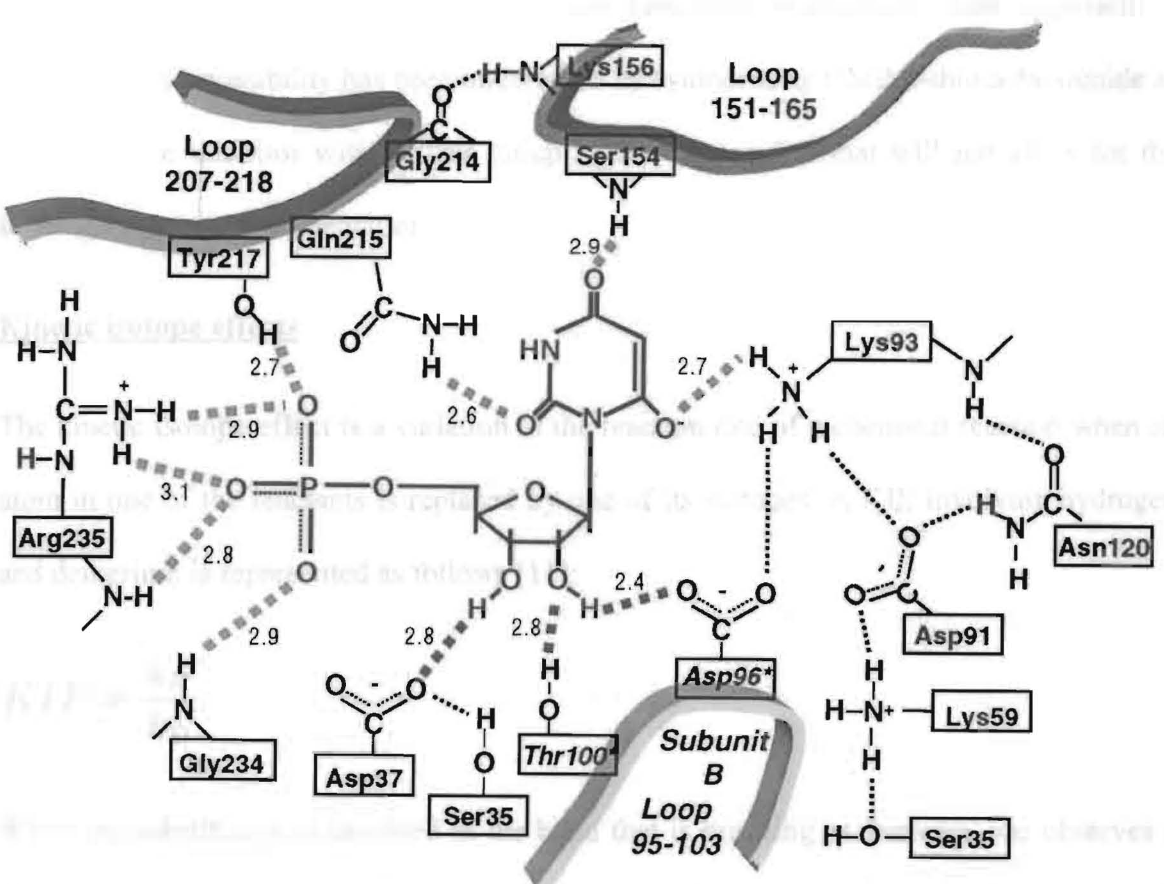


Figure 1-6: Binding of BMP in the yeast ODCase active site Miller and Wolfenden, Reference [3].

the peptide amide of Ser-154 contacts O-4 of BMP. The remaining residues of Loop 151-165 provide a "hydrophobic umbrella" covering O-4 and C-5 of the pyrimidine ring. The fourth set of interactions involves a mobile domain, Loop 207-217, which is disordered in the free enzyme but well organized in the inhibitor-enzyme complex.

Our lab is concerned with investigating whether OMP could bind in the reverse orientation since the inhibitor used in obtaining the crystal structure, BMP, is highly symmetrical and

could easily bind in the reverse orientation and fit perfectly in the enzyme active site. If OMP binds in the reverse orientation, Lys93 of yeast ODCase or Lys73 of *E-coli* could protonate the O2 to favor the zwitterrion formation mechanism. One approach to addressing this possibility has been undertaken by synthesizing UMP-6-thiocarboxamide as an alternative inhibitor with bulkier group at the C6 position that will not allow for the binding in the reverse orientation.

Kinetic isotope effects

The kinetic isotope effect is a variation in the reaction rate of a chemical reaction when an atom in one of the reactants is replaced by one of its isotopes. A KIE involving hydrogen and deuterium is represented as follows [11]:

$$KIE = \frac{k_H}{k_D}$$

When the substitution is involved in the bond that is breaking or forming, one observes a rate called primary isotope effect. When the substitution is not involved in the bond that is breaking or forming, one may still observe a smaller rate change, termed a secondary isotope effect. Isotopic rate changes are most pronounced when the relative mass change is greatest. Isotopic rate changes are most pronounced when the relative mass change is greater; therefore, one expects a higher isotope effect involving hydrogen and deuterium as compared to ^{12}C and ^{13}C. The rate of a reaction involving a C-H bond is typically 6 to 10 times faster than the corresponding C-D bond, whereas a ^{12}C reaction is only ~1.04 times faster than the corresponding ^{13}C reaction.

Isotope effects are determined by measuring the change in isotopic composition of either the substrate or product in an enzyme catalyzed reaction. The isotope content is measured

for a partial reaction and a complete reaction. The relationship of the isotope content in the partial reaction with the complete reaction gives us the isotope effect. If a substrate contains a mixture of unlabeled and labeled substrate species is converted into product, the isotopic compositions of the substrate species change in the course of the reaction because the labeled and unlabeled substrates react at a different speed.

ODCase from *Saccharomyces ceriviseae* displays ^{13}C kinetic isotope effect of 1.0247 ± 0.0008 at 25 °C . The kinetic isotope effect is dependent on temperature, pH and glycerol content [12]. The variation of the isotope effect as a function of the reactants can be used to determine the kinetic mechanism while the pH variation of the isotope effect can be used to determine the stickiness of the reactants and which portions of the reactant mechanism are pH dependent while the size of primary and secondary intrinsic isotope effects can be used to determine transition state structure [13].

There are various ways in which isotope effects can be to probe enzymatic mechanisms; the power of this method is fully realized when isotope effects are measured as a function of something else that modulates catalytic activity [14]. The isotope effect studies can be used in identifying the rate-limiting step, kinetic mechanisms and characterization of iso-mechanisms with solvent isotope effects.

A minimal kinetic model of for OMP decarboxylase is shown below:

$$E + S \rightleftharpoons ES \rightleftharpoons ES^{*} \longrightarrow EP + CO_2$$

Figure: 1-7

ES^* represents an enzyme –substrate complex in which the substrate has undergone a chemical step responsible for catalysis [15]. This denotes the enzyme bound to zwitterionic intermediate immediately proceeding decarboxylation.

Chapter 2: Isotope effect studies of labeled and unlabeled OMP

Introduction

Current studies of ODCase mechanism of action favor the zwitterionic intermediate formation in the decarboxylation of OMP. The process involves protonation of O2 and elimination of CO_2 to generate a nitrogen ylide [16]. It is proposed that lysine (Lys93 in yeast and Lys73 in *E. coli*) protonate O2 in OMP to generate a zwitterion followed by decarboxylation. [17]. My first project involves synthesis of ^{18}O at O2 to see the isotope effects of this modification on decarboxylation of ODCase.

This project involved the synthesis of naturally occurring OMP, ^{13}C depleted OMP, and double labeled OMP with ^{13}C at C6 and ^{18}O at O2 and reacting with ODCase from *E. coli.* The synthesized double labeled substrate (OMP) with the ^{13}C labeled carbon at the carbonyl carbon at position 6 (the key position) and ^{18}O labeled at position 2 (indicator position) is run to partial completion using the *E . coli* system. A complete reaction is also run to get the ^{13}C content in both reactions. The complete reaction is expected to contain more ^{13}C content compared to the partial reaction. This is because ^{13}C is slow at the bond breaking step compared to ^{12}C. A separate reaction is run for the naturally occurring OMP and the isotope effects are determined. It is proposed that there will be a decrease in the isotope effect in the double labeled substrate as compared to the naturally occurring OMP. The reason for a lower isotope effect is because ^{18}O is involved in an equilibrium isotope effect before decarboxylation. The value of the equilibrium isotope effect is equal to the ratio of the two kinetic isotope effects k_l / k_o (k_l -labeled substrate kinetic isotope effect; k_o-unlabeled substrate kinetic isotope effect).

An even lower equilibrium isotope effect is expected at lower pH values because lower pH favors the formation of the zwitterionic intermediate.

Materials and methods

The double labeled OMP was synthesized using the following synthetic scheme:

Figure 2-1: Synthesis of [^{8}O2and ^{13}C-*carboxyl*] OMP

Synthesis of 2-ethylthiouracil and (^{18}O2)-Uracil

Synthesis of 2-ethylthiouracil was performed by mixing 1 g of 2-thiouracil and 1.2 g (0.625mL) of iodoethane and dissolving in 15 mL DMF (Figure 1-8, step 1). Reaction was heated gently at 60°C and let to run overnight. The reaction was monitored using TLC. The best solvent system for TLC was determined to be 40% hexane/60% ethylacetate. 2-ethylthiouracil had a lower RF value compared to the other alkylated by-products. 2-ethylthiouracil was purified using silica gel chromatography using 40% hexane/60%

ethylacetate. Two separate reactions were conducted to generate 0.23 g and 0.17 g respectively.

HCl gas was bubbled through $H_2{}^{18}O$ (> 97% ^{18}O, Cambridge Isotope Laboratories) until the resulting solution was thoroughly acidic. 0.320 mg of 2-ethylthiouracil was added to 1 mL of acidified $H_2{}^{18}O$; the reaction vial was sealed, vented with a hypodermic needle, and heated at 70°C (Reaction step 2, Figure 1-8) Evolution of gas (thioethane) was observed, and the heating continued until gas evolution ceased. Samples of the reaction mix were analyzed by TLC using 5% methanol and 95% ethylacetate with 2-ethylthiouracil and uracil standards; heating was resumed until no more 2-ethylthiouracil could be observed in the reaction mix. Uracil had a lower RF value compared to 2-ethylthiouracil. 0.15 g of ^{18}O labeled uracil was synthesized with a 70% yield. The resulting uracil was analyzed using a Bruker Esquire mass spectrometer, under electrospray ionization conditions for anion detection. ^{18}O labeled uracil was successfully detected with a mass of 113.1 compared to 111 of unlabeled uracil.

Utilization of UPRTase for synthesis of (^{18}O2) -UMP

UPRTase was used in the synthesis of (^{18}O2) UMP as described by Jensen, *et al.* [18]. Initial reaction was performed using 1 mg unenriched uracil , 25 mM Tris HCl , pH 8.6 , 5 mM $MgCl_2$, 2 mg BSA[19]. The reaction was started by addition of 5.5 mg of phosphoribosyl pyrophosphate (PRPP) and protein carried out at 37°C in a water bath while shaking. [^{18}O2] UMP synthesis (Step 3, Figure 1-8) was carried out in a similar fashion only that the reaction was scaled up by using a total volume of 6 mL. The reaction was monitored by HPLC at 270 nm with the reaction times ranging between 0 and 80 min. The retention times for uracil and UMP were 4.8 and 6 min respectively. [^{18}O2]- uracil and

[^{18}O2] UMP retention times were 4.8 and 7.0 min respectively. Once the reaction was 95% complete it was frozen to ensure that [^{18}O2] UMP was not degraded by the crude protein. Purification was performed by anion exchange chromatography. After purification [^{18}O2] UMP was characterize by Bruker Esquire mass spectrometer to ensure retention of isotope enrichment.

Syntheses of 5-Br –UMP , 6-cyano-UMP , [^{18}O2] -5-Br-UMP, and [^{18}O2,^{13}C-*cyano*]-6-CN-UMP

Synthesis of 5-Br-UMP and 6-CN-UMP were performed using to the protocols described by Ueda and colleagues [20]. Synthesis of 5-Br–UMP was carried out dissolving 1:1 molar ratio of UMP [^{18}O2]-UMP and Br_2 in pyridine/acetic acid mixture of 2:1 volume ratio. The reaction was let to run overnight while stirring. The reaction was monitored by HPLC and was 100% complete by the next day. The retention time for 5-Br-UMP was 8.3 min. The solvent was evaporated and water was added and evaporated three times. The product was purified using anion exchange chromatography using a gradient of 0 to 0.8 M ammonium bicarbonate

5-Br-UMP containing ammonium bicarbonate added to an acid washed resin to get rid of ammonium bicarbonate. This was done till a pH of 2 was attained. The solvent was then evaporated to dryness then dissolved in DMF and evaporated to dry syrup. 2:1 molar ratio of Na^{13}CN and Br-UMP was used and dissolved in about 15mL of DMSO. The reaction

was let to proceed for 4 days. The product was purified using anion exchange chromatography with a gradient of 0 to 0.4 t0 0.8 ammonium bicarbonate. The product was analysed using HPLC with a retention time of 9.3min.

Conversion of [^{18}O2,^{13}C-*cyano*]-6-CN-UMP to [^{18}O,^{13}C-*carboxyl*]-OMP

Test reactions using small quantities of the double labeled 6-CN-UMP were used to ensure that the ^{18}O label is not exchanged at higher pH using NaOH. The reaction was performed by addition of NaOH to a final concentration of 0.5 M and let to stir for 4 days at room temperature. The reaction was monitored by HPLC and proceeded to 97% completion. Retention time of the double labeled OMP was 7.3 min. The nucleotide was desalted using acid washed resin.

Synthesis of [^{13}C carboxyl-depleted] OMP

Figure 2-2: Synthesis of isotopically depleted OMP

2 g of UMP was dissolved in a pyridine/acetic acid mixture 2:1 volume ratio. 0.656g of Bromine was added the reaction was stirred and let to run overnight at room temperature.

The reaction was monitored by HPLC and was 100% complete by the next day. The solvent was evaporated to dryness, water was added and evaporated to dryness. The process was repeated three times. Water was added and the sample was loaded onto anion exchange column. A gradient of 0 to 0.8 M ammonium bicarbonate was used to purify the product. The product was quantified using UV-VIS spectrophotometry with a percentage yield of 95%. The product was desalted using an acid washed resin till the pH was 2 and the solvent evaporated.

5-Br-UMP was dissolved in DMF and evaporated to dry syrup. 20 mL of DMSO was added and 260 mg of $K^{12}CN$ was added. The reaction was stirred and let to run for 2 days. After 2 days 65.11 mg of $K^{12}CN$ was added. The reaction was let to run for another 3 days. The product was loaded onto anion exchange column and a 0 to 0.8M ammonium bicarbonate used to elute the product. Fractions containing 95% ^{13}C depleted 6-cyano-UMP were retained. Product was quantified using UV-VIS spectroscopy with 62% yield. The product was desalted using acid washed resin

^{13}C-depleted 6-cyano-UMP was mixed with NaOH to a final pH of 0.5 M NaOH. The reaction was let to stir at room temperature for 4 days and monitored using HPLC. The reaction was about 97% complete and was quantified using UV-VIS spectroscopy.

Unlabelled OMP was also synthesized using the same protocol as above only that unlabelled NaCN was used instead of $K^{12}CN$ while synthesizing 6-CN-UMP.

Measuring isotope effects on enzymatic ODCase decarboxylation reactions

Trial reactions were run using a 1/10 scale of enzyme and substrate in order to determine the amount of enzyme needed to convert 20-30% of OMP to UMP [21].This was done by

using 0.5mL of 10mM OMP and 0.5mL 7.0 MOPS buffer. A partial reaction was run by using 5 mL of 10 mM OMP and 5 mL MOPS buffer. The mixture was transferred into a clean reaction flask and caped with a rubber septum. A needle was inserted in a septum, immersed in the substrate buffer mixture and connected to the nitrogen tank. The solution was degassed for 1 hour. An enzyme solution of 1 mg/mL enzyme was degassed for about 5 min. The solution was then placed in a water bath at 25 ^{o}C and 200 μL of enzyme was added for a partial reaction and 1.2 mL of enzyme added for complete reaction. Partial reactions were let to run for 5-7 min while complete reactions were let to run for 20 min. The reaction was quenched by adding 600 μL 1 M H_2SO_4. The sample was frozen with liquid nitrogen and connected to the CO_2 distillation set up and distillation performed. Once the CO_2 distillation was complete it was analyzed for ^{13}C isotopic content. Reactions were run for naturally occurring OMP and isotopically prepared OMP. The isotopically prepared OMP isotope content was adjusted to a near natural abundance before the values were used to calculate the enzymatic kinetic effects.

Results and Discussion

Isotopically labeled [$^{18}O_2$]-uracil was successfully synthesized; this was confirmed by LC-MS (Figure 1-11) with about 98% enrichment. We experienced some problem in dissolving uracil in 30% methanol which is the solvent for MS analysis, however, there was enough sample dissolved for analysis.

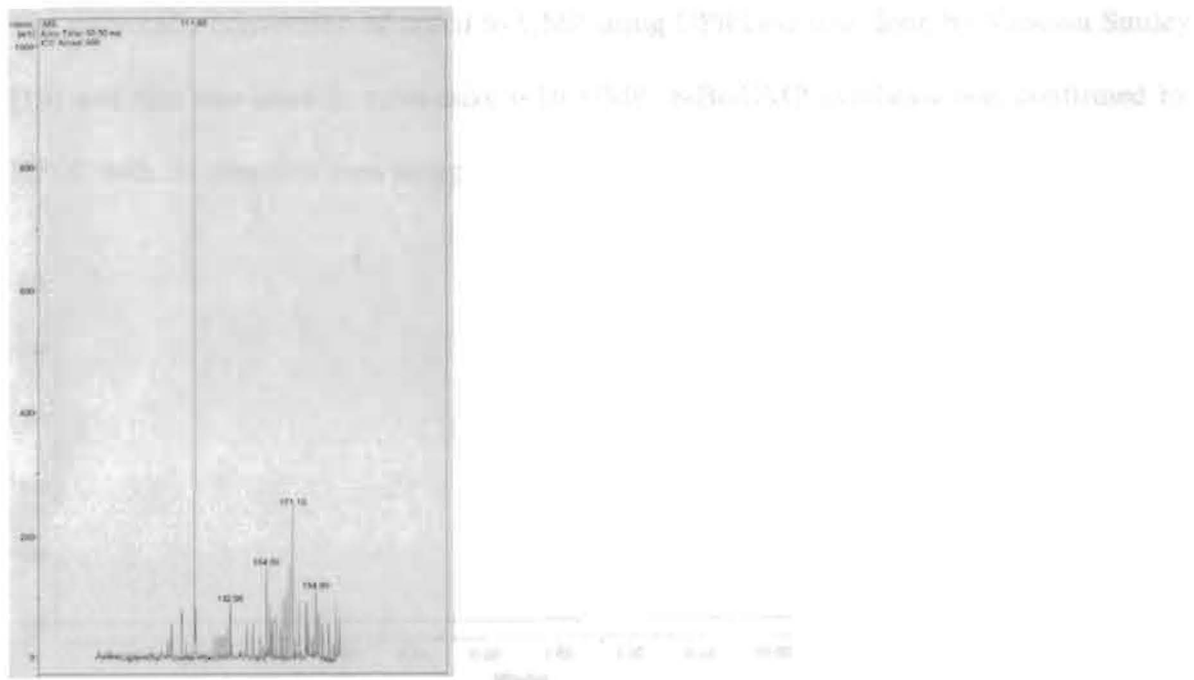


Figure 2-3: LC-MS data of natural abundance uracil

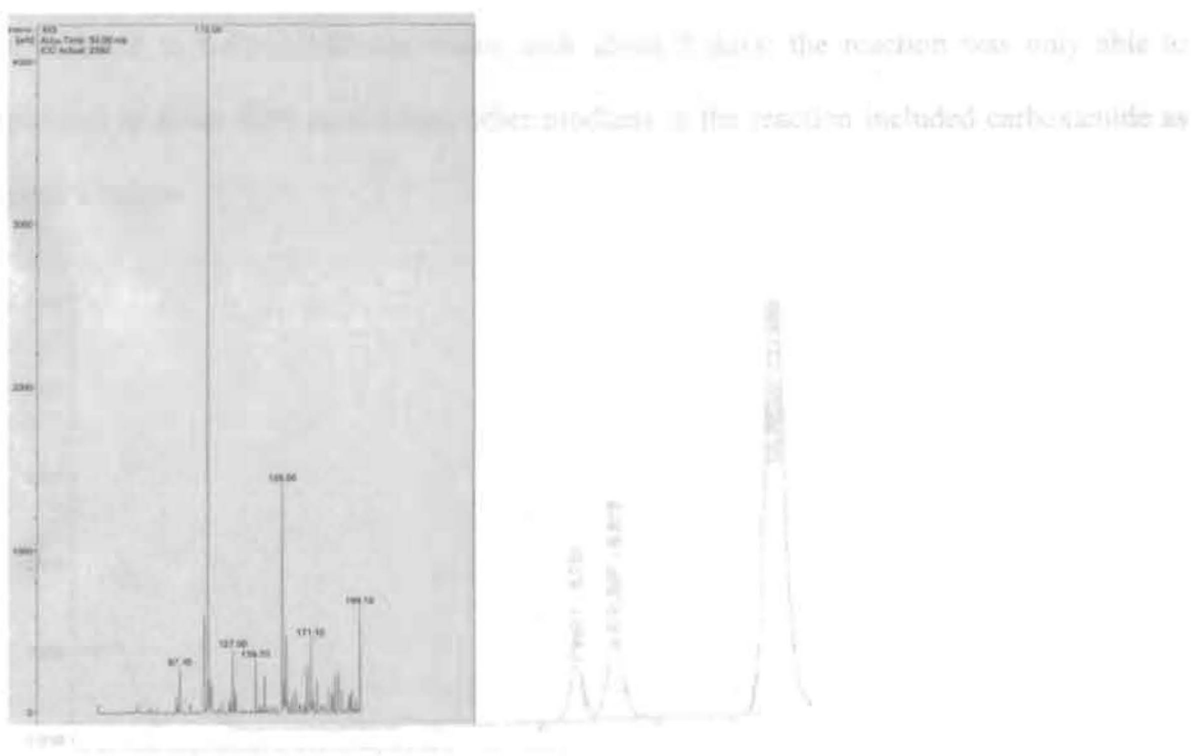


Figure 2-4: LC-MS data of [^{18}O2] labeled uracil

The enzymatic conversion of uracil to UMP using UPRTase was done by Vanessa Smiley [19] and this was used to synthesize 6-Br-UMP. 6-Br-UMP synthesis was confirmed by HPLC with its retention time being about 7.8 minutes.

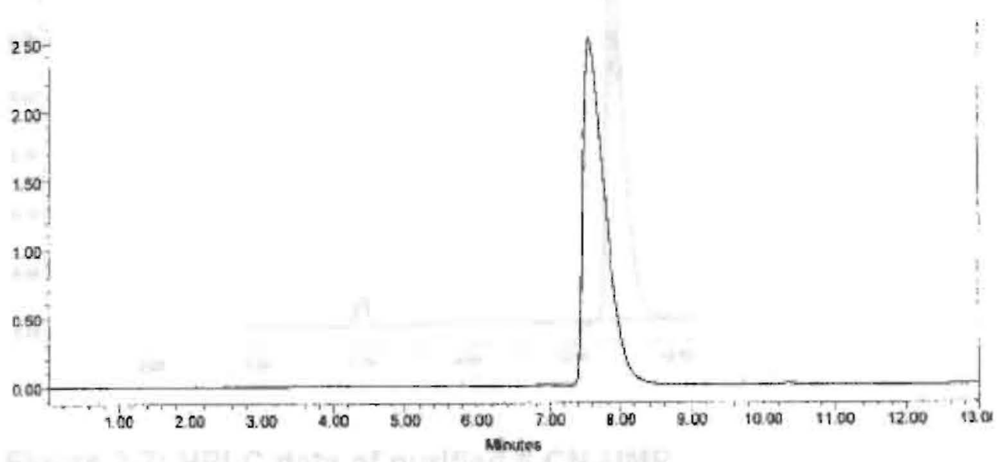


Figure 2-5: HPLC data of 5-Br-UMP retention time

6-Br-UMP to 6-CN-UMP conversion took about 5 days; the reaction was only able to proceed to about 80% conversion, other products in the reaction included carboxamide as peak 1 below.

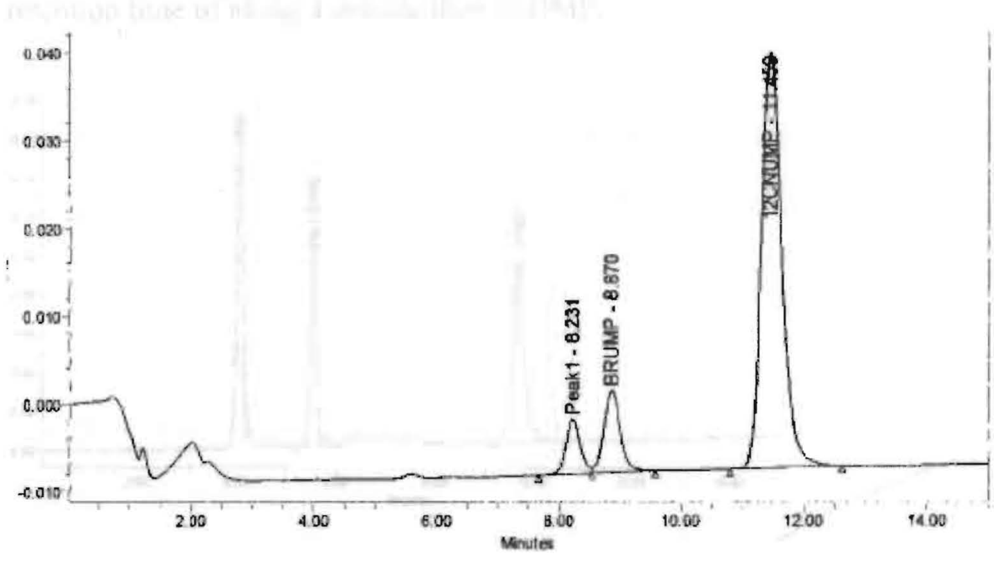


Figure 2-6: HPLC data showing conversion of 5-Br-UMP to 6-CN-UMP

The product was purified using ion exchange chromatography and fractions containing > 95% CN-UMP were pooled together. 6-CN-UMP retention time was about 10.8 minutes.

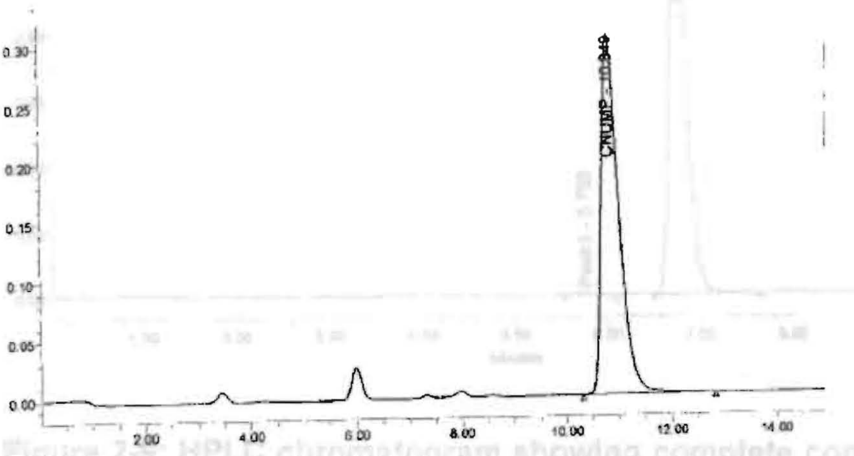


Figure 2-7: HPLC data of purified 6-CN-UMP

Conversion of 6-CN-UMP to OMP was initially heated but this yielded to unwanted products so the reaction was let to run at room temperature. The reaction proceeded to about 99% after 6 days, retention time for OMP was about 6.59 minutes. The reaction normally involves the conversion of 6-CN-UMP to an unknown compound with a retention time of about 4 minute then to OMP.

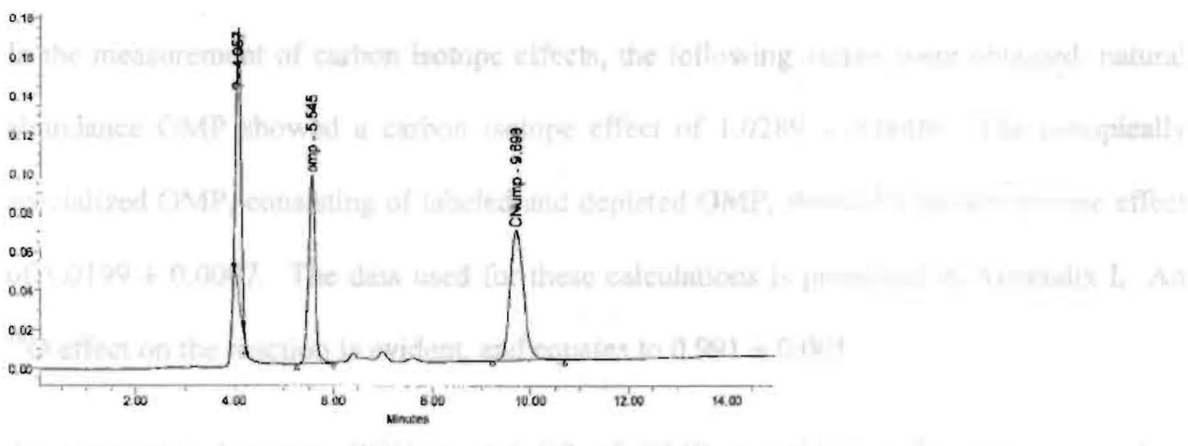


Figure 2-8: HPLC chromatogram showing partial conversion of 6-CN-UMP to OMP

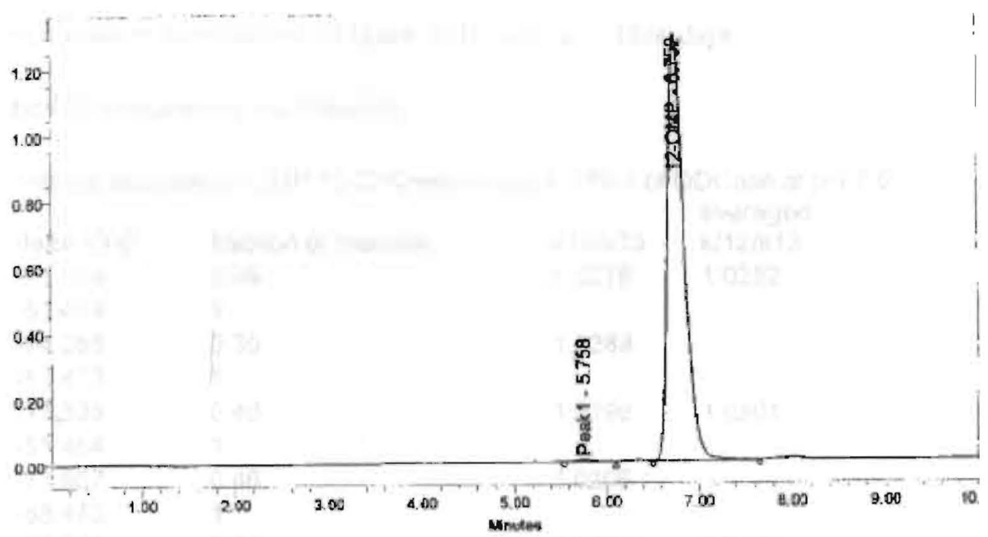


Figure 2-9: HPLC chromatogram showing complete conversion of 6-CN-UMP to OMP

Initially the isotope analysis of complete reactions of isotopically specialized OMP gave large positive values. This was attributed to an excess amount of ^{13}C labeled OMP. The proportion of labeled and unlabeled OMP was adjusted to give an isotopic value that was close to natural abundance.

In the measurement of carbon isotope effects, the following values were obtained: natural abundance OMP showed a carbon isotope effect of 1.0289 ± 0.0009. The isotopically specialized OMP, consisting of labeled and depleted OMP, showed a carbon isotope effect of 1.0199 ± 0.0007. The data used for these calculations is presented in Appendix I. An ^{18}O effect on the reaction is evident, and equates to 0.991 ± 0.001.

An interaction between ODCase and O2 of OMP is evidenced by this data. Any mechanism for the decarboxylation of OMP by ODCase must include some participation of O2 of the substrate. The only mechanism thus far suggested in the literature is the O2

protonation mechanism (Figure 1-4, path a). This data appears to be strong evidence for the O2 protonation mechanism.

Natural abundance OMP 13-C Kinetic Isotope Effect of ODCase at pH 7.0

delta 13-C	fraction of reaction	k12/k13	averaged k/12/k13		
-71.324	0.39	1.0276	1.0282		
-51.454	1				
-74.085	0.39	1.0288			
-53.473	1				
-71.335	0.46	1.0296	1.0301		
-51.454	1				
-73.957	0.46	1.0306			
-53.473	1				
-70.674	0.32	1.0271	1.0277		
-50	1				
-73.087	0.32	1.0283			
-51.597	1				
-69.1	0.4814	1.0304	1.0295		
-49.064	1				
-70.306	0.4814	1.0286			
-51.428	1			Average:	1.0289
					± 0.0009

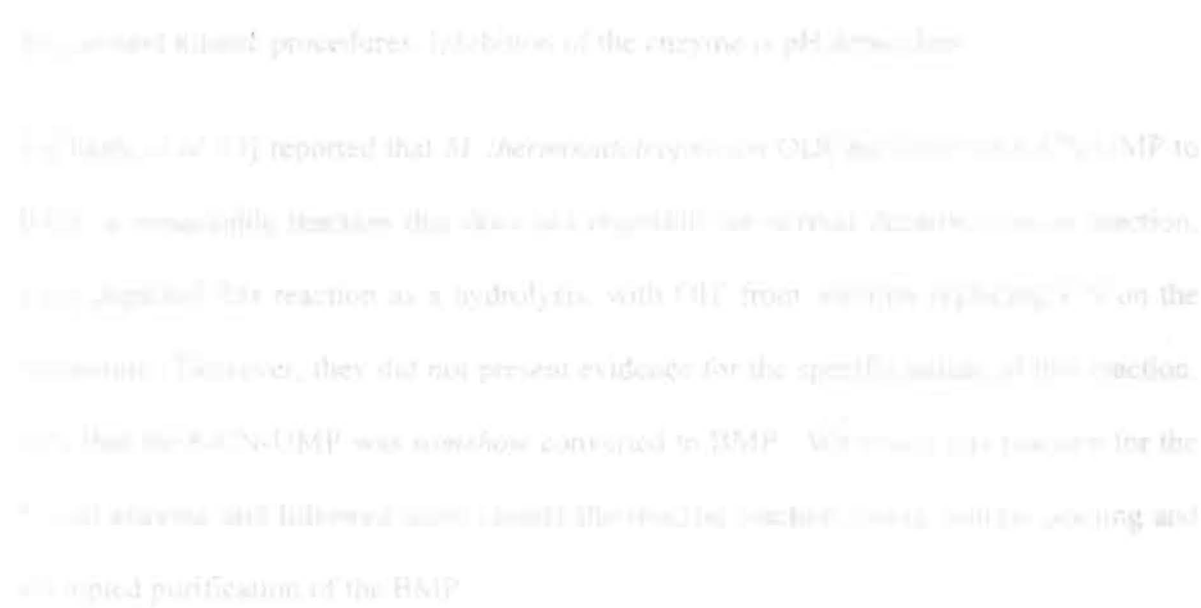
Isotopically specialized OMP 13-C Kinetic Isotope Effect of ODCase at pH 7.0

delta 13-C	fraction of reaction	k12/k13	averaged k12/k13		
-34.33	0.30012	1.0212	1.02115		
-17.337	1				
-33.906	0.30012	1.0211			
-16.954	1				
-34.33	0.30012	1.0207	1.02085		
-17.705	1				
-33.906	0.30012	1.021			
-17.073	1				
-25.88	0.597	1.0202	1.01995		
-13.8	1				
-25.403	0.597	1.0197			
-13.67	1				
-52.301	0.44	1.019	1.01935		
-39.036	1				
-53.408	0.44	1.0197			
-39.629	1				
-53.324	0.37	1.0192	1.0192		
-39.036	1				
-53.889	0.37	1.0192		Average:	1.0199
-39.036	1				± 0.0007

Table 1-1: Compilation of Carbon Isotope effect data

delta value for 13-C depleted OMP	-382.346 -368.465
delta value for CO_2 reference to reference	0.104 0.133
delta value for natural abundant bicarbonate	-5.842 -6.529
delta value for spiked bicarbonate	44.831 43.953

Table 1-2: Data showing consistency of isotope ratio measurements, using standard samples.



Chapter 3 – The ODCase Reaction with 6-CN-UMP Inhibitor

Introduction

5'-Phospho-*b*-D-ribofuranosyl (barbituric) acid is a potent inhibitor of ODCase; it binds to ODCase 100000 times as strongly as does the substrate. It has shown to be a potent inhibitor of ODCase from rat brain with a K_i of $4.1x10^{-9}$ [22]. BMP is an extraordinarily powerful inhibitor of yeast enzyme, binding so tightly that the stoichiometric enzyme-inhibitor complex can be purified by gel-filtration [23]. 6-azauridine 5'-phosphate is also a known potent inhibitor of ODCase, its inhibition constant is $4.6x10^{-4}$M when determined by standard kinetic procedures. Inhibition of the enzyme is pH dependent.

Fujihashi *et al.* [9] reported that *M. thermoautotrophicum* ODCase converts 6-CN-UMP to BMP, a remarkable reaction that does not resemble the normal decarboxylation reaction. They depicted this reaction as a hydrolysis, with OH^- from solution replacing CN on the nucleotide. However, they did not present evidence for the specific nature of this reaction, only that the 6-CN-UMP was *somehow* converted to BMP. We tested this reaction for the *E. coli* enzyme and followed more closely the specific reaction, using isotope labeling and attempted purification of the BMP.

Materials and methods

Purification of E. coli ODCase from Overproducing strain

Incubation of *E. coli*

BL21 cells carrying plasmid pCal-EC ODCase were obtained using sterile technique tooth pick a colony and transferred the toothpick into a 50mL volume LB. The culture was

incubated for 30 minutes while shaking at 37°C. 50 uL of 50 mg/mL ampicillin was added and returned the culture to incubate overnight. The next day 10 mL of the culture was transferred to each liter of LB, then added 1 mL of 0.5 mg/ml ampicillin and returned the culture to incubate at 37°C while shaking for three hours. The culture was removed from the incubator and 600 uL of IPTG was added and let to incubate at 37°C with shaking for 4 hours. The cells were collected by centrifugation at 5000rpm for ten minutes and discarded the supernatant.

Preparation of lysate

The cells were resuspended in Lysis buffer (50 mM potassium phosphate, 10% glycerol, 5 mM DTT and protease inhibitors PMSF, leupeptin and pepstatin 1:200 ratio) that was equal to twice the mass of the cell pellet. The cells were transferred to a bead beater containing 0.1 mm glass beads; the container had 2/3 full of cell suspension making sure no air bubbles were introduced. Using ice to cool the contents of the container, the bead beater was pulsed for three one-minute periods, interspaced with 2-3 minutes periods of cooling the contents. The glass beads were allowed to settle and transferred the lysate into Corex centrifuge tubes. The contents were span at10000 rpm for 20 minutes. The supernatant was removed and discarded the pellet.

Ammonium sulphate fractionation of ODCase

The volume of the lysate was determined and a 40% addition of Ammonium sulphate; for each 10 mL of lysate 2.26 grams of Ammonium sulphate was added. The amount of ammonium sulphate was added slowly, about 0.5g every minute, with thorough stirring in the refrigerator. After all the ammonium sulphate was added stirring continued for 30 minutes. . The solution was transferred to Corex centrifuge tubes and centrifuged at

10000rpm for 15 minutes. The supernatant was transferred to another graduated cylinder and measured the volume then transferred into a clean beaker. A second addition of 70% ammonium sulphate was accomplished by adding 1.87 g of ammonium sulfate for every 10 mL of lysate, after all the salt had been added stirring continued for 30 minutes. The solution was transferred Corex centrifuge tubes and span at 10000 rpm for 15 minutes. This time the pellet was retained and 5 mL of pH 7.4 dialysis buffer (50 mM Tris, 10% glycerol, 5 mM DTT). The solution was inserted into dialysis tubing and immersed in one liter of dialysis buffer. The container was set on a stir plate in cold and let to stir overnight.

Affinity gel chromatography of ODCase

A well packed Affigel blue column was washed with 200 mM NaCl, then restored with dialysis buffer with no salt. The protein was transferred into the column with the chromatography pump turned on at 2mL per minute which allowed the protein to be taken up the column. Once all the protein had been taken up the transfer inlet tubing was dipped into a solution containing dialysis buffer. Fractions were collected and fractions checked for presence of proteins using 0.5 mL of Bradford reagent and 20 μL of each fraction. Fractions containing protein were obviously blue. Fractions were collected till no more protein was observed. The inlet tube was then transferred into a solution containing 200 μM UMP. More fractions were collected this time ODCase is present in the fractions. Normally about 15-20 fractions of ODCase were collected. The fractions were pooled and quantified using Bradford assay. The purity of protein was the checked by SDA-PAGE analysis. Normally about 30- 50 mg of protein were purified for a 4 liter LB media.

Effect of freezing and thawing on the ODCase activity

The purpose of this experiment is to figure out whether ODCase activity is diminished over time when frozen and thawed. This is important for our subsequent enzymatic reactions which needed a large amount of enzyme preparations which was to be frozen and thawed for the different enzymatic reactions.

Two samples of freshly prepared ODCase were stored at 4 degrees and -20 degree C and their specific activity measured over 5 days. Each time the enzyme was thawed and its enzyme activity assessed.

ODCase reaction with 6-CN-UMP

3mL reactions using different concentrations of ODCase (125 μM, 280 μM and 625 μM) were incubated with 10 times the concentration of 6-CN-UMP (1.25mM, 2.8mM and 6.25mM) in a 15 mL conical tube. The reaction was let to run for five days while checking the enzyme activity. Once the activity of the enzyme was diminished to its minimum, ethanol was added (final concentration = 50%) to denature the protein and release the bound inhibitor. The sample was then centrifuged for 30 min at 13000rpm and the supernatant was removed. The supernatant was evaporated to near-dryness, filtered, and injected onto the HPLC to purify the different peaks that arise. All the peaks were collected into plastic conical tubes and evaporated; this was done several times to get rid of all the ammonium bicarbonate. Each fraction was then analyzed using ESI Bruker Esquire LC-MS.

To test whether or not the oxygen atom added in the 6-CN-UMP to BMP reaction was derived from water, reactions were run as above with 63% $H_2{}^{18}O$, by adding $H_2{}^{18}O$ (> 97% ^{18}O, Cambridge Isotope Laboratories

ODCase reaction with added O_2

Test reactions were conducted to ascertain whether oxygen is involved in the ODCase/6-CN-UMP reaction. Three 3mL reactions containing 0.225 uMoles ODCase and 2.25 uMoles of 6-CN-UMP were placed in a 25mL Erlenmeyer flask, capped with a rubber septum. Flask 1 was bubbled with nitrogen gas for 10 minutes; flask 2 was bubbled with oxygen for 10 minutes while flask 3 was let to progress at room conditions. The enzyme activity was monitored for 52 hrs.

The effects of *repeated* oxygen exposure were assessed in the following experiment. Four 3-mL reactions were set containing 0.225 μMoles ODCase and 2.25 μMoles 6-CN-UMP in a 25-mL septum-capped Erlenmeyer flask. Flask 1 was let to run under atmosphere, flask 2 was bubbled with oxygen once(at 0hrs), flask 3 was bubbled with oxygen three times at 10-hour intervals(at 0hrs, 10hrs, and 20hrs) and flask 4 was bubbled with oxygen 4 times at 10 hour intervals(at 0hrs, 10hrs,20hrs and 30hrs). The enzyme activity was monitored throughout the reaction time until the enzyme activity had reached a minimum.

Results and Discussion

ODCase activity is not diminished over a 5 day period whether stored at 4° C or at -20°C. Freezing and thawing the enzyme does not adversely affect the enzyme activity. This allowed us to prepare large amounts of protein, freeze and thaw while maintaining the enzyme activity.

Enzyme activity in nmol $min^{-1}ug^{-1}$

Day	Stored at 4° C	Stored at -20° C
0	50	50
4	76	69
5	76	89

Table 3-1: Measurement of ODCase activity in samples stored at 4 °C or -20 °C.

From mass spec data, there was further evidence that BMP with a molecular mass of 339 (monoanion) was one of the products of the 6-CN-UMP reaction with ODCase.

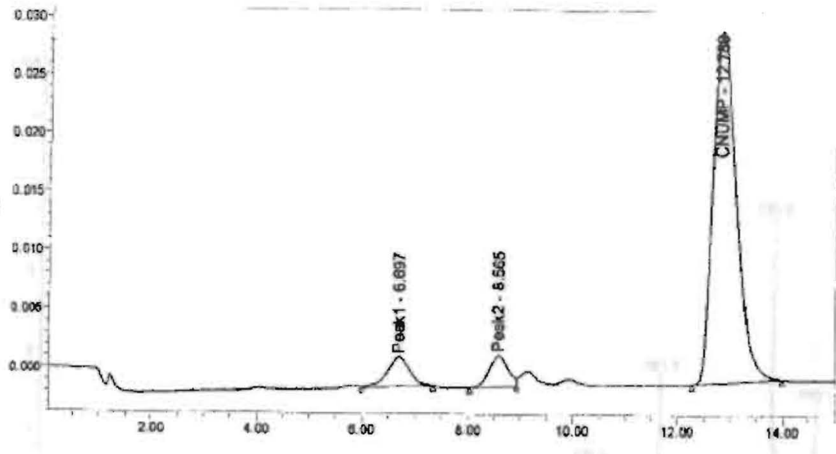


Figure 3-1: Unreacted 6-CN-UMP

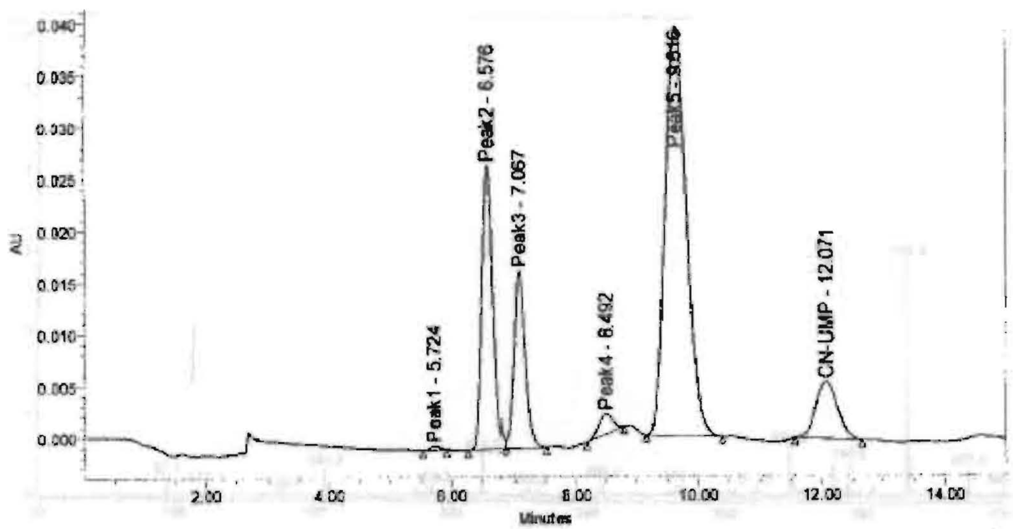


Figure 3-2: ODCase-CN-UMP reaction after 3 days. Protein was precipitated and the supernatant was concentrated and analyzed.

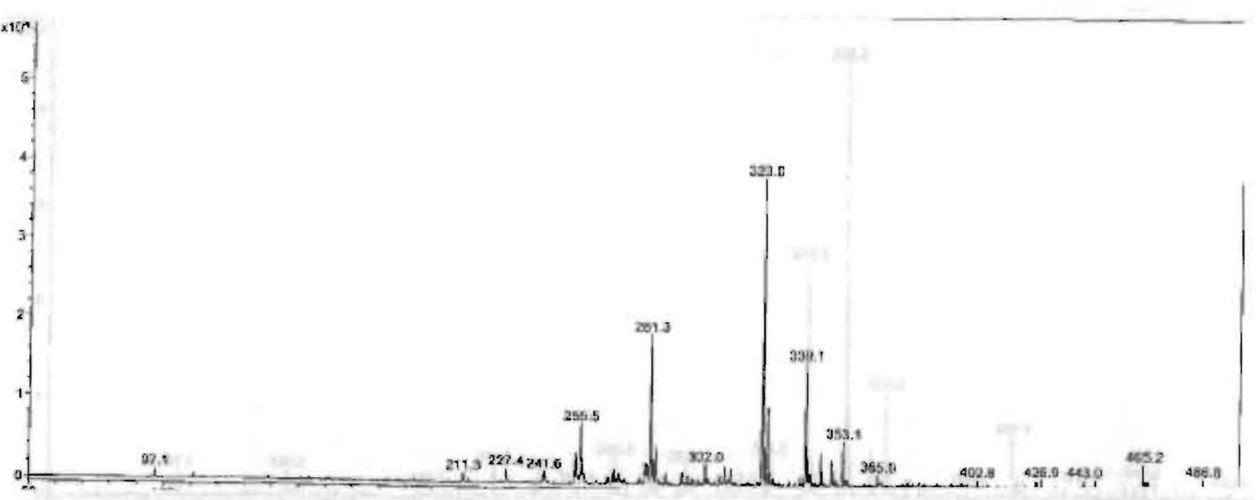


Figure 3-3: Mass spec analysis of peak 2 of 6-CN-UMP reaction

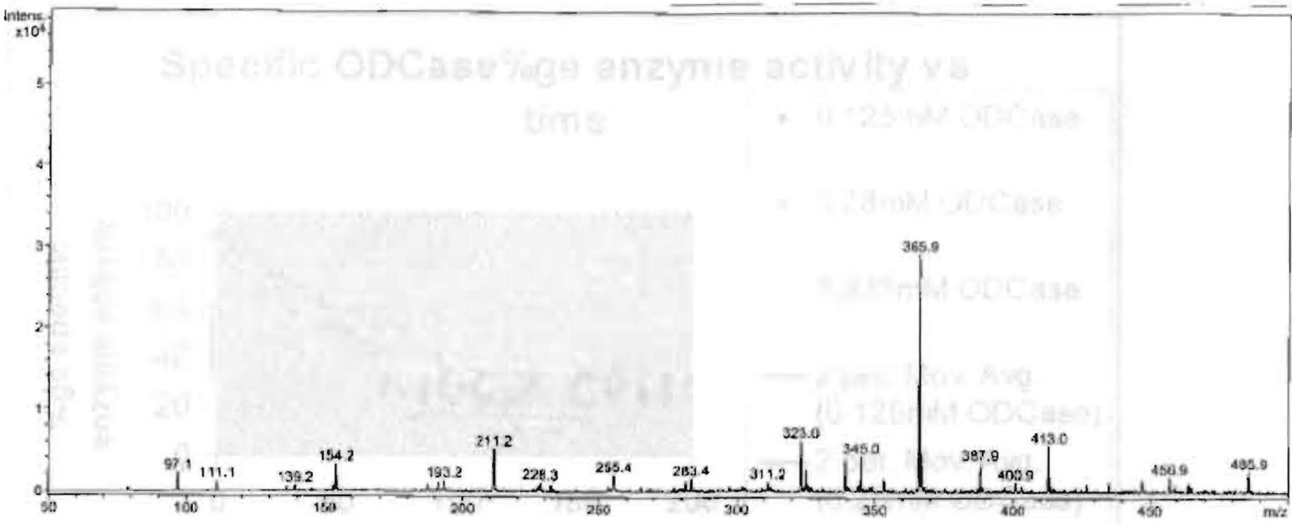


Figure 3-4: Mass spec analysis of peak 3 of 6-CN-UMP reaction

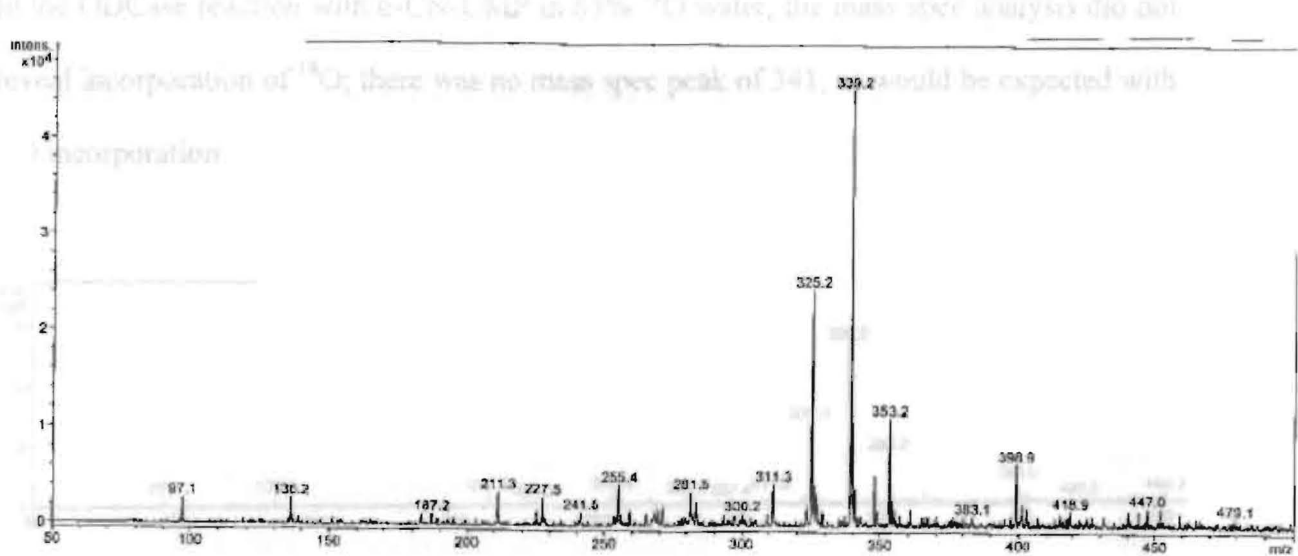


Figure 3-5: Mass spec analysis of peak 5 of 6-CN-UMP reaction. The large amount of mass = 339 is evidence for BMP in this fraction.

ODCase activity decreased over the time period of the reaction with 6-CN-UMP, in a manner similar to that seen previously [9]

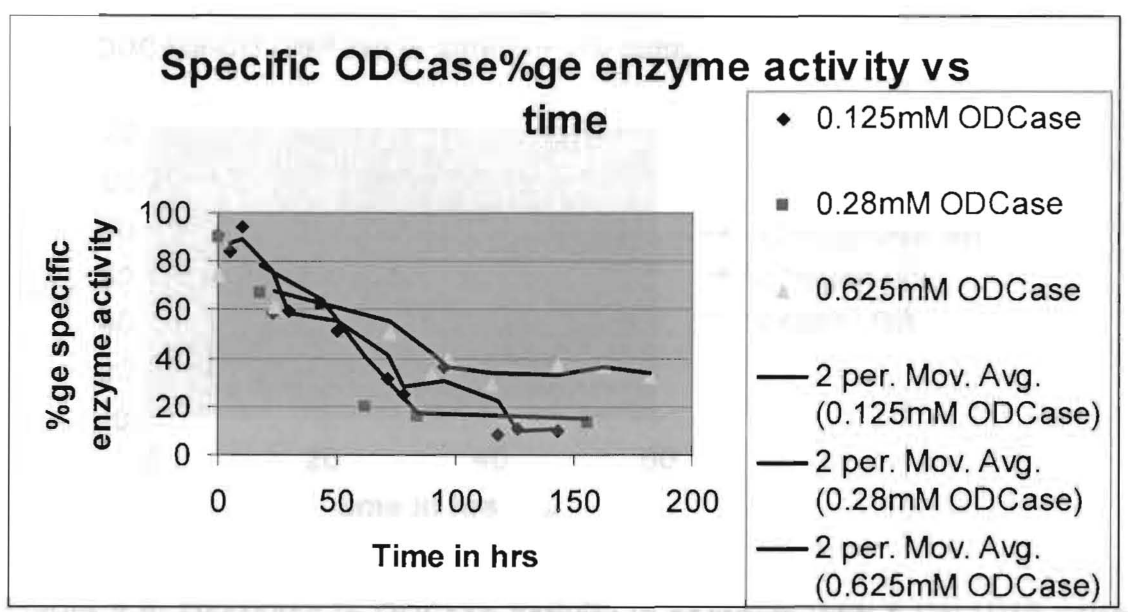


Figure 3-6: Time-dependent decrease in ODCase activity upon reaction with 6-CN-UMP.

In the ODCase reaction with 6-CN-UMP in 63% ^{18}O water, the mass spec analysis did not reveal incorporation of ^{18}O; there was no mass spec peak of 341, as would be expected with ^{18}O incorporation.

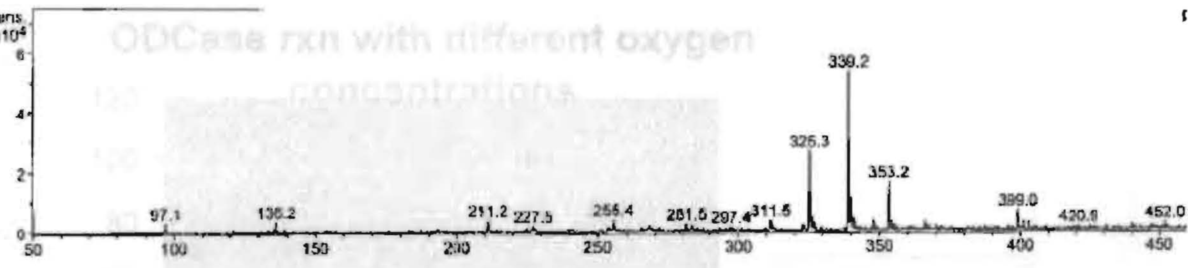


Figure 3-7: Mass spec analysis of BMP from reaction of ODCase and 6-CN-UMP in 63% $H_2{}^{18}O$.

The reaction bubbled with oxygen showed a faster rate of diminishing activity while the reactions bubbled with nitrogen and under atmosphere showed pretty much similar diminished rate of enzyme activity. However the specific enzyme activity did not diminish to zero, but instead only to about 10%.

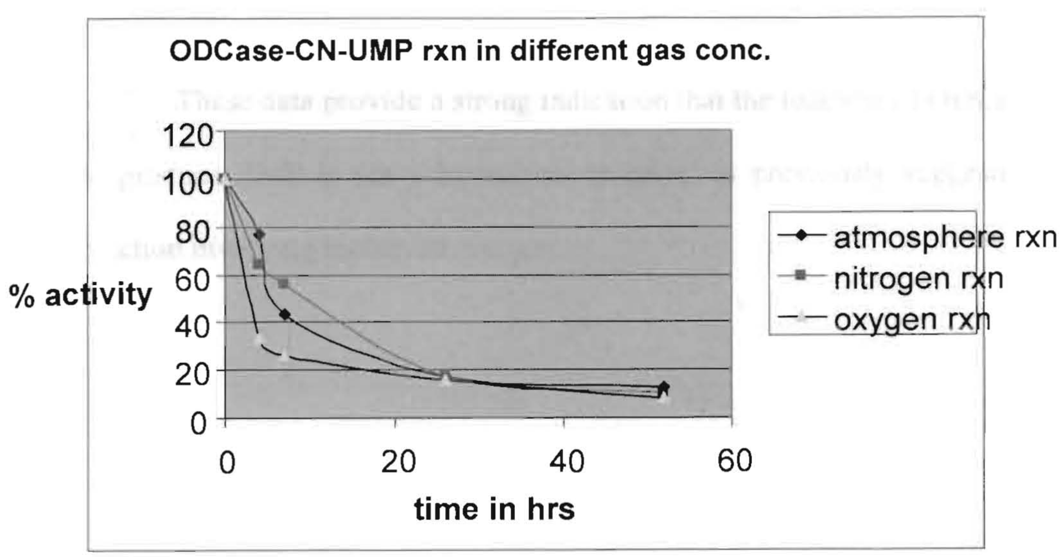


Figure 3-8: Decrease in ODCase activity in samples with 6-CN-UMP exposed to O_2, N_2 or atmosphere.

Multiple exposures of the ODCase/6-CN-UMP reaction mixtures to oxygen resulted in complete decrease of ODCase activity to essentially zero.

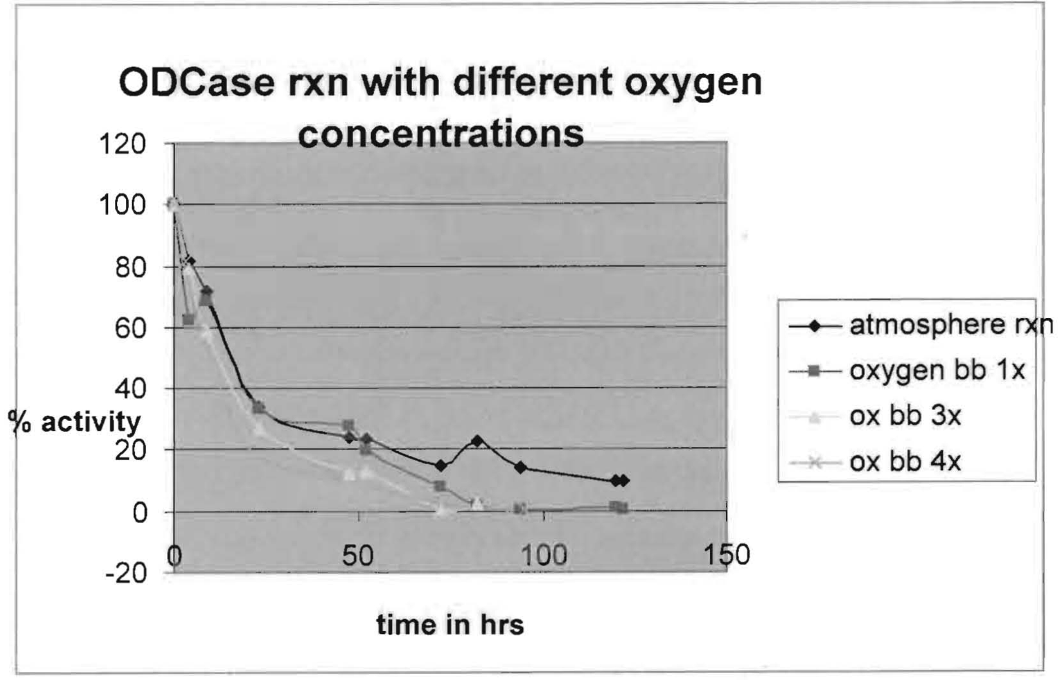


Figure 3-9: Decrease in ODCase activity in samples with 6-CN-UMP exposed multiple to O_2.

These data provide a strong indication that the reaction of ODCase with 6-CN-UMP to produce BMP is not a hydrolysis reaction, as previously suggested [9]but instead is reaction involving molecular oxygen.

Chapter 4:

Final Discussion on Isotope effect studies of labeled and unlabeled OMP and the ODCase Reaction with 6-CN-UMP Inhibitor

Isotope effect studies of labeled and unlabeled OMP

Current studies of ODCase mechanism of action favor the zwitterionic intermediate formation in the decarboxylation of OMP. The process involves protonation of O2 and elimination of CO_2 to generate a nitrogen ylide . It is proposed that lysine (Lys93 in yeast and Lys73 in *E. coli*) protonate O2 in OMP to generate a zwitterion followed by decarboxylation.

We successfully synthesized a specially labeled and unlabeled OMP and ran partial and complete reactions to obtain kinetic isotope values. We predicted that Lys73 in *E. coli* is involved in protonation of O2 in OMP to generate a zwitterion followed by decarboxylation . If ^{16}O2 was replaced with ^{18}O2 then there will be a longer equilibration time required during the protonation step leading to a drop in kinetic isotope effect. This turned out to be the case leading us to conclude that protonation of O2 and formation of the zwitterionic species occur prior to decarboxylation.

Discussing reaction ODCase with 6-CN-UMP Inhibitor

In previously conducted experiments [9] of ODCase from *M. thermoautotrophicium* incubated with 6-CN-UMP, the enzyme showed diminished activity due to the formation of BMP, a potent inhibitor. We were able to get similar results with ODCase from *E. coli.* LC-MS data conclusively suggested the formation of BMP. However it was previously

suggested that the formation of BMP occurs is a hydrolysis reaction. After incubating the ODCase and 6-CN-UMP reaction in 63% ^{18}O labeled water there was no indication of ^{18}O incorporation indicating that hydrolysis reaction might not be taking place. When the reaction was bubbled with O_2 there was a significant decrease in enzyme activity suggesting that molecular oxygen might be involved. The diminishing of the enzyme activity proceeded to an almost zero value unlike the atmosphere run reactions which were only diminished to about 10%. We hope in future to conclusively figure out the source of oxygen incorporated; this could be done using NMR reactions in figuring out the fate of cyano group eliminated from 6-CN-UMP.

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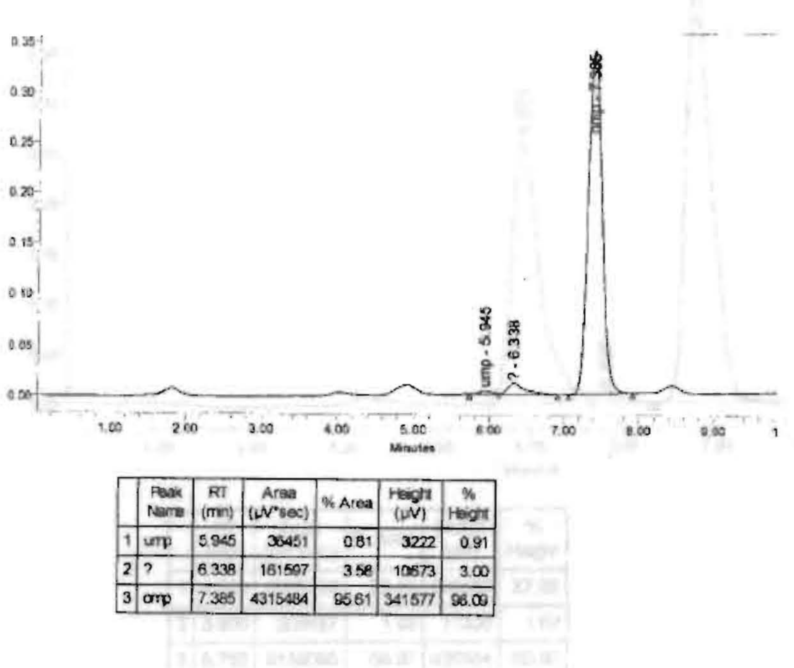
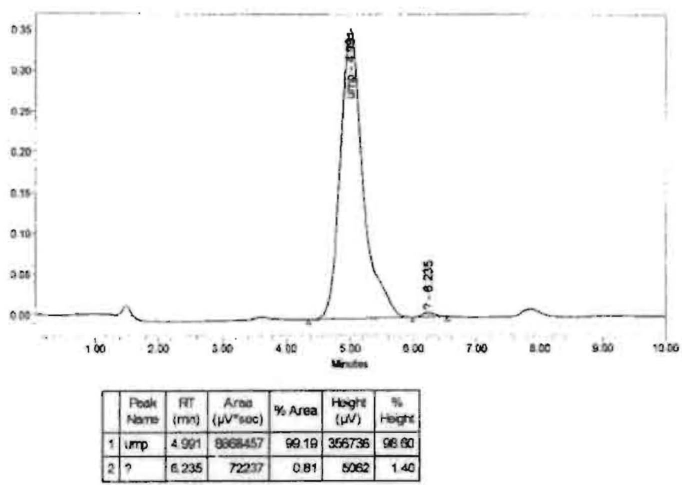
APPENDIX I – RAW DATA FOR THE CALCULATION OF CARBON ISOTOPE EFFECTS IN THE ODCASE REACTION



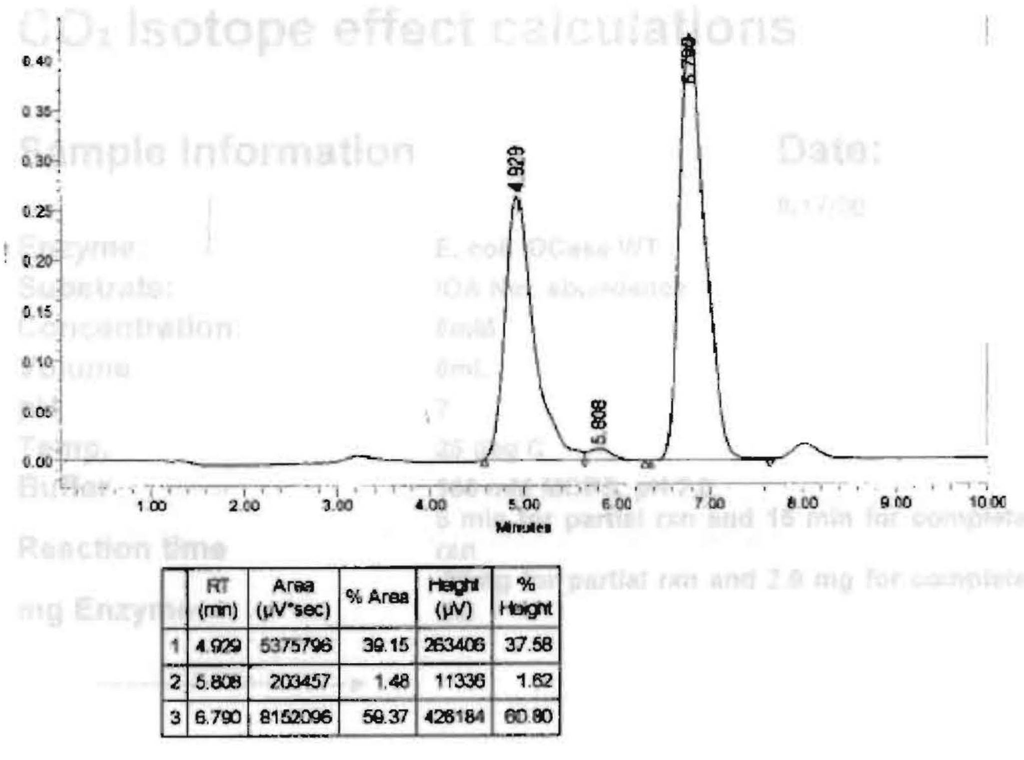
	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	5.945	36451	0.81	3222	0.91
2	?	6.338	161597	3.58	10673	3.00
3	omp	7.385	4315484	95.61	341577	96.09

Figure AI - 1: HPLC chromatogram showing unreacted OMP



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	4.991	8868457	99.19	356736	98.60
2	?	6.235	72237	0.81	5062	1.40

Figure AI - 2: HPLC chromatogram showing complete conversion of natural abundant OMP to UMP reaction 1



	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	4.929	5375796	39.15	263406	37.58
2	5.808	203457	1.48	11336	1.62
3	6.790	8152096	59.37	428184	60.80

Figure AI - 3: HPLC chromatograph showing partial conversion of OMP reaction 1

CO_2 Isotope effect calculations

Sample Information

Date: 8/17/06

Enzyme:	**E. coli IDCase WT**
Substrate:	**IOA Nat. abundance**
Concentration:	**5mM**
Volume	**8mL**
pH	**7**
Temp.	**25 deg C**
Buffer	**100 mM MOPS, pH 7.0**
Reaction time	**5 min for partial rxn and 15 min for complete rxn**
mg Enzyme	**.25mg for partial rxn and 2.0 mg for complete rxn**

→

Fraction reaction (0.0 - 1.0) 0.39

→

Delta value (partial rxn) -71.324

→

Delta value (100% conversion) -51.454

→

Isotope effect 1.0277

→

User W.O.W

Table AI – 1: Isotope effect calculation for natural abundance OMP reaction 1

CO_2 Isotope effect calculations

Sample Information

Date:

Enter date 8/17/2006

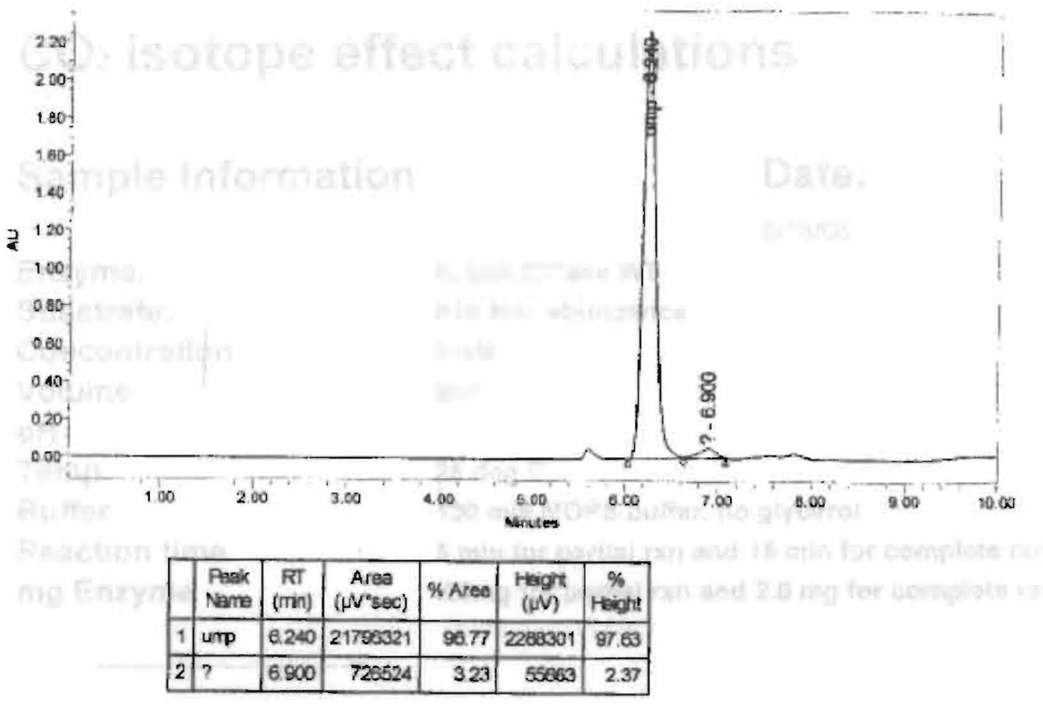
Enzyme:	E. coli ODCase WT
Substrate:	OMP Nat. abundance
Concentration:	5 mM
Volume	10 mL
pH	7
Temp.	25 C
Buffer	MOPS buffer PH 7.0
Reaction time	5 min for part rxn and 15 min for complete rxn
mg Enzyme	.25 mg for part rxn 1mg complete rxn

Fraction reaction (0.0 - 1.0)	0.39	**1000 + δ**
Delta value (partial rxn)	-74.085	925.915
Delta value (100% conversion)	-53.473	946.527
Isotope effect ⟶	1.0288	

User

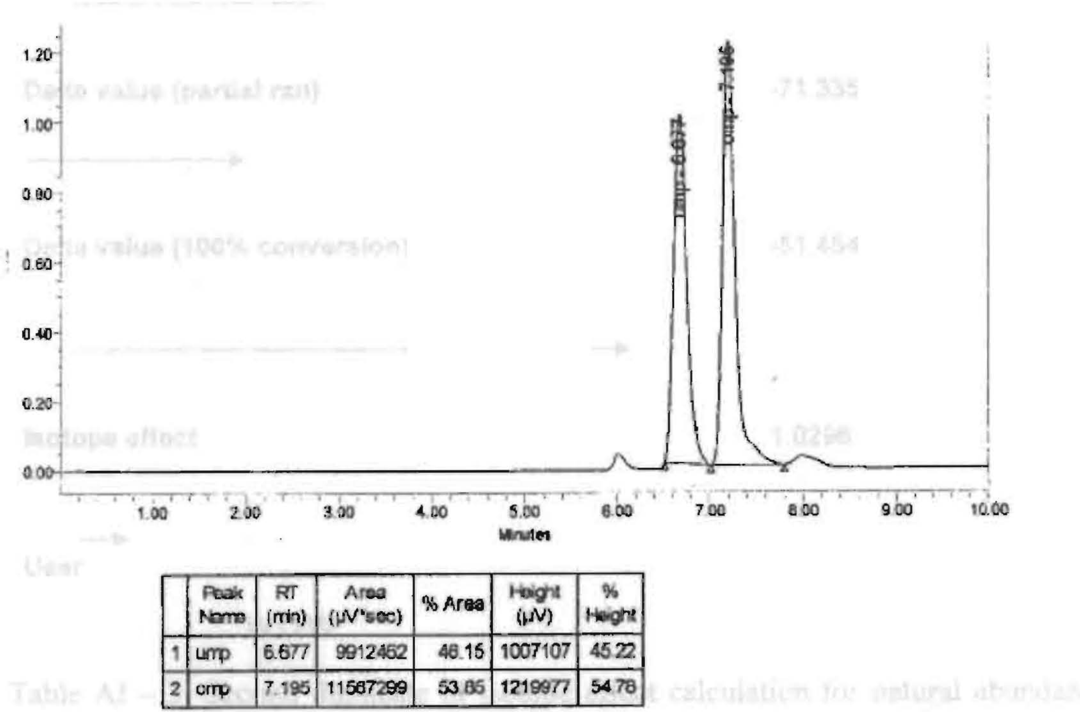
⟶ W.O.W

Table AI – 2: First repeat of isotope effect calculation for natural abundance OMP reaction



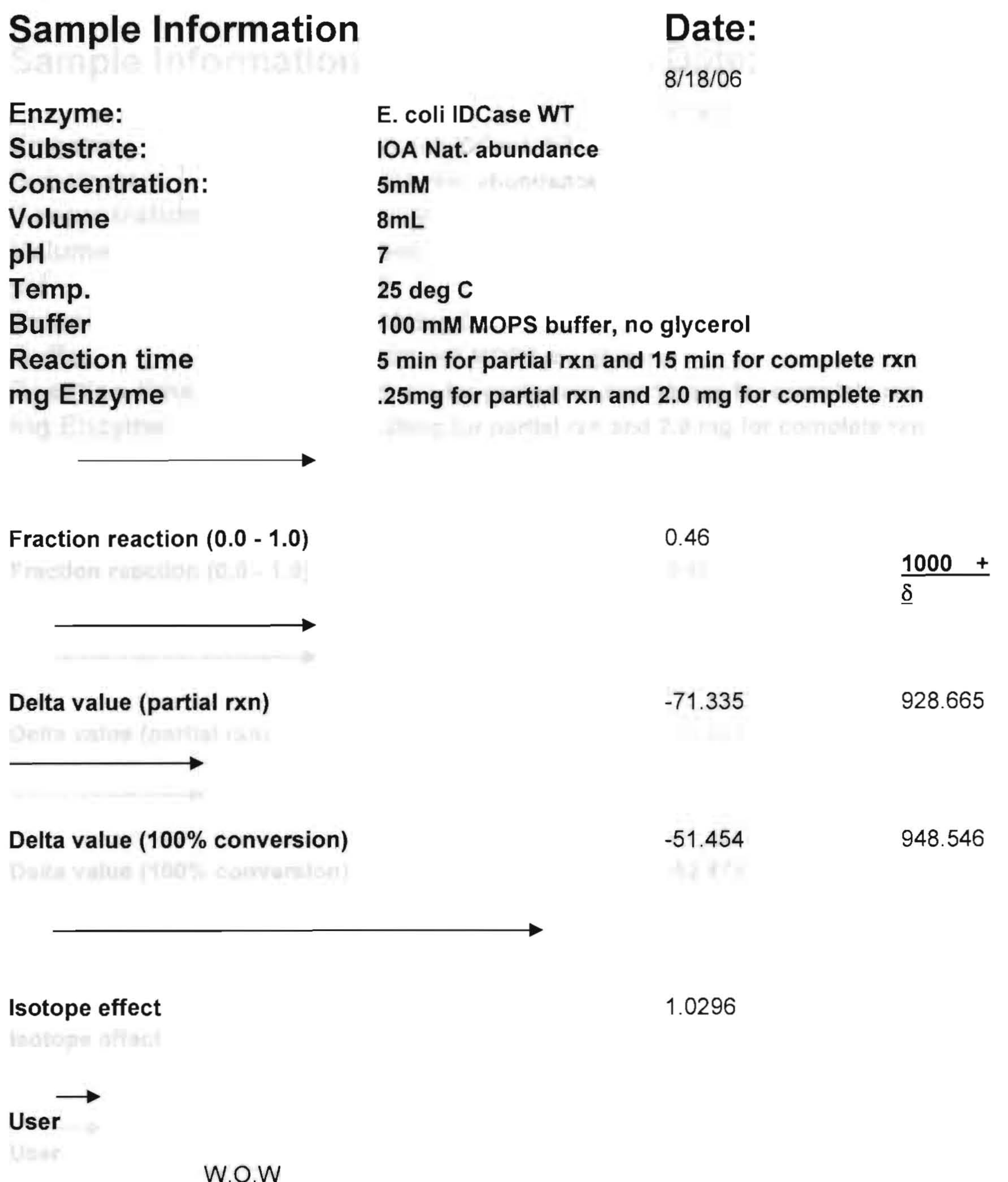
	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	6.240	21796321	96.77	2288301	97.63
2	?	6.900	728524	3.23	55663	2.37

Figure AI – 4: HPLC chromatogram showing complete reaction 2



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	6.677	9912462	46.15	1007107	45.22
2	omp	7.195	11567299	53.85	1219977	54.78

Figure AI – 5: HPLC chromatogram showing partial reaction 2



CO_2 Isotope effect calculations

Sample Information

Date: 8/18/06

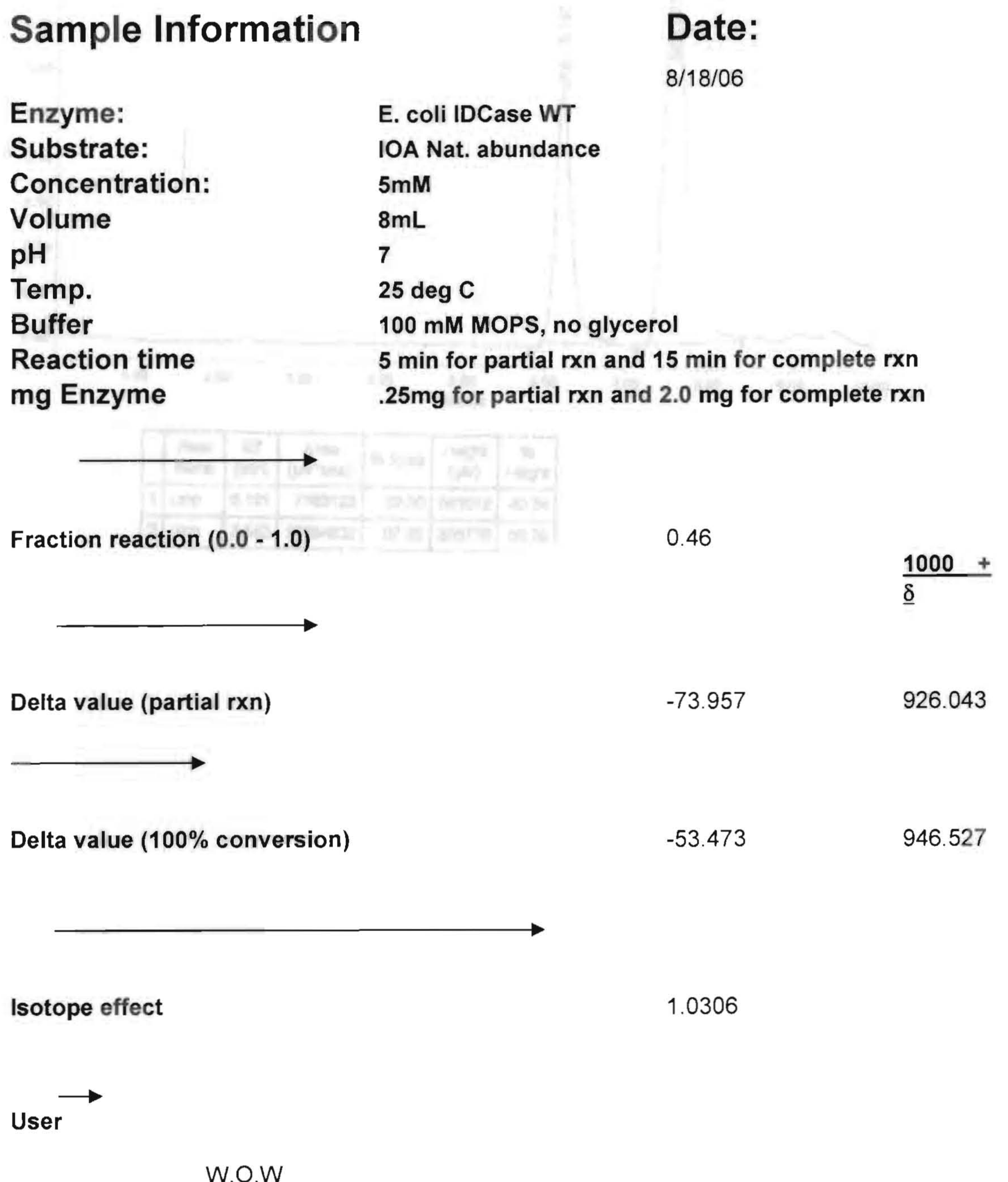
Enzyme:	E. coli IDCase WT
Substrate:	IOA Nat. abundance
Concentration:	5mM
Volume	8mL
pH	7
Temp.	25 deg C
Buffer	100 mM MOPS buffer, no glycerol
Reaction time	5 min for partial rxn and 15 min for complete rxn
mg Enzyme	.25mg for partial rxn and 2.0 mg for complete rxn

		$1000 + \delta$
Fraction reaction (0.0 - 1.0)	0.46	
Delta value (partial rxn)	-71.335	928.665
Delta value (100% conversion)	-51.454	948.546
Isotope effect	1.0296	

User

W.O.W

Table AI – 3: Second duplicate of isotope effect calculation for natural abundance OMP reaction2.



CO_2 Isotope effect calculations

Sample Information

Date:
8/18/06

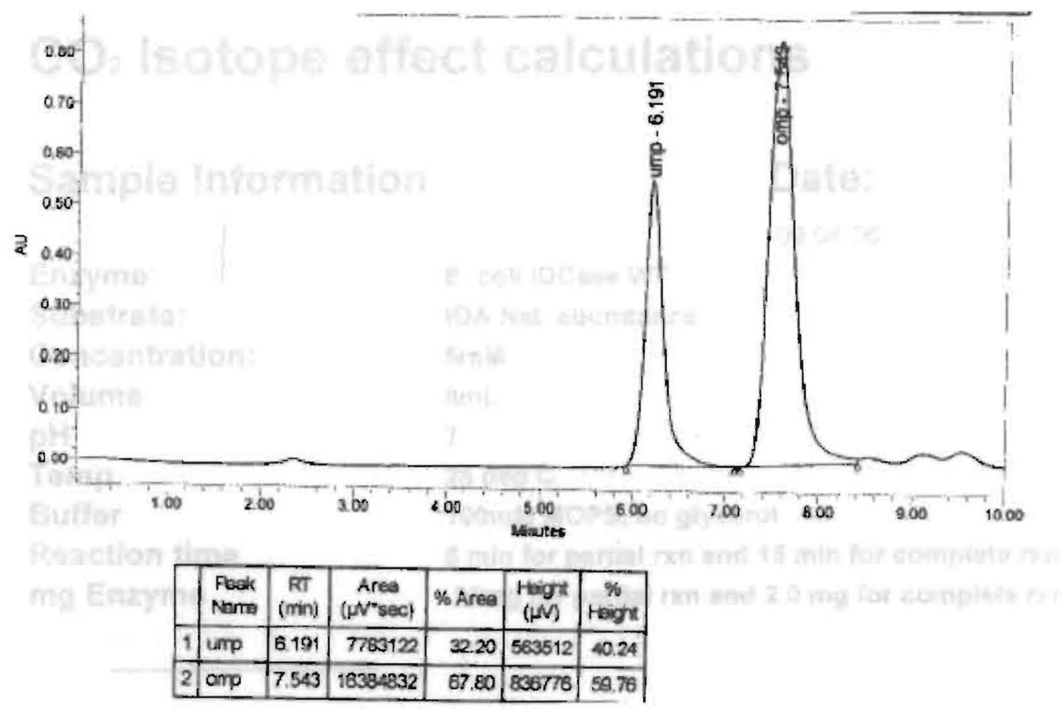
Enzyme:	E. coli IDCase WT
Substrate:	IOA Nat. abundance
Concentration:	5mM
Volume	8mL
pH	7
Temp.	25 deg C
Buffer	100 mM MOPS, no glycerol
Reaction time	5 min for partial rxn and 15 min for complete rxn
mg Enzyme	.25mg for partial rxn and 2.0 mg for complete rxn

Fraction reaction (0.0 - 1.0)	0.46	1000 + δ
Delta value (partial rxn)	-73.957	926.043
Delta value (100% conversion)	-53.473	946.527
Isotope effect	1.0306	

User

W.O.W

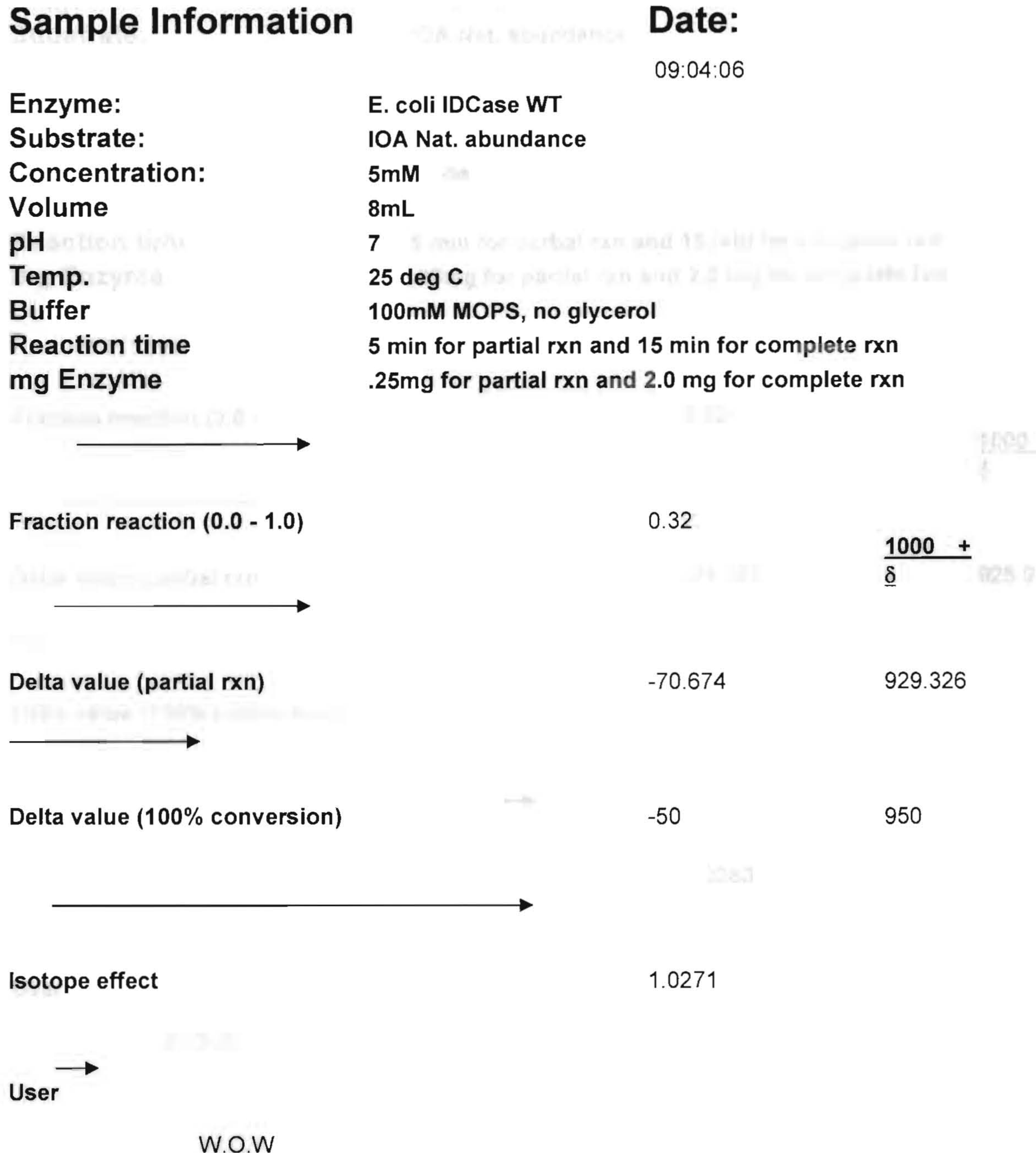
Table AI – 4: Second repeat of isotope effect calculation for natural abundance OMP reaction 2.



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	6.191	7783122	32.20	563512	40.24
2	omp	7.543	16384832	67.80	836776	59.76

Figure AI – 6: HPLC chromatogram showing partial reaction 3

CO_2 Isotope effect calculations



Sample Information

Date: 09:04:06

Enzyme:	**E. coli IDCase WT**
Substrate:	**IOA Nat. abundance**
Concentration:	**5mM**
Volume	**8mL**
pH	**7**
Temp.	**25 deg C**
Buffer	**100mM MOPS, no glycerol**
Reaction time	**5 min for partial rxn and 15 min for complete rxn**
mg Enzyme	**.25mg for partial rxn and 2.0 mg for complete rxn**

		1000 + δ
Fraction reaction (0.0 - 1.0)	0.32	
Delta value (partial rxn)	-70.674	929.326
Delta value (100% conversion)	-50	950
Isotope effect	1.0271	

User

W.O.W

Table AI – 5: Third repeat of isotope effect calculation for natural abundance OMP reaction 3

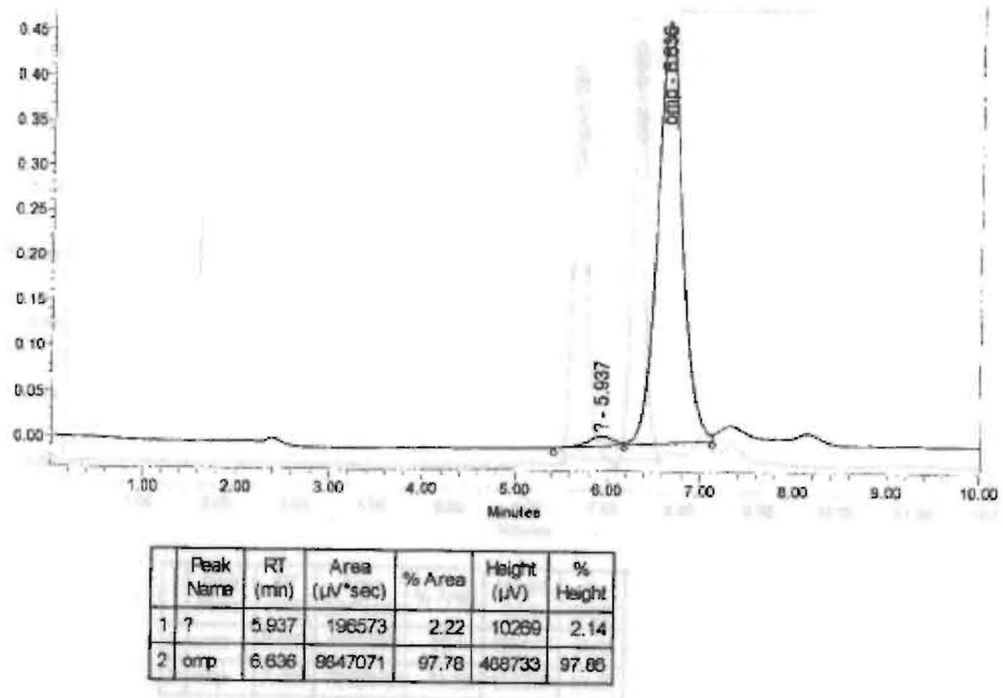
CO_2 Isotope effect calculations

09:04:06

Enzyme:	**E. coli IDCase WT**
Substrate:	**IOA Nat. abundance**
Concentration:	**5mM**
Volume	**8mL**
pH	**7**
Temp.	**25 deg C**
Buffer	**100 mM MOPS, no glycerol**
Reaction time	**5 min for partial rxn and 15 min for complete rxn**
mg Enzyme	**.25mg for partial rxn and 2.0 mg for complete rxn**

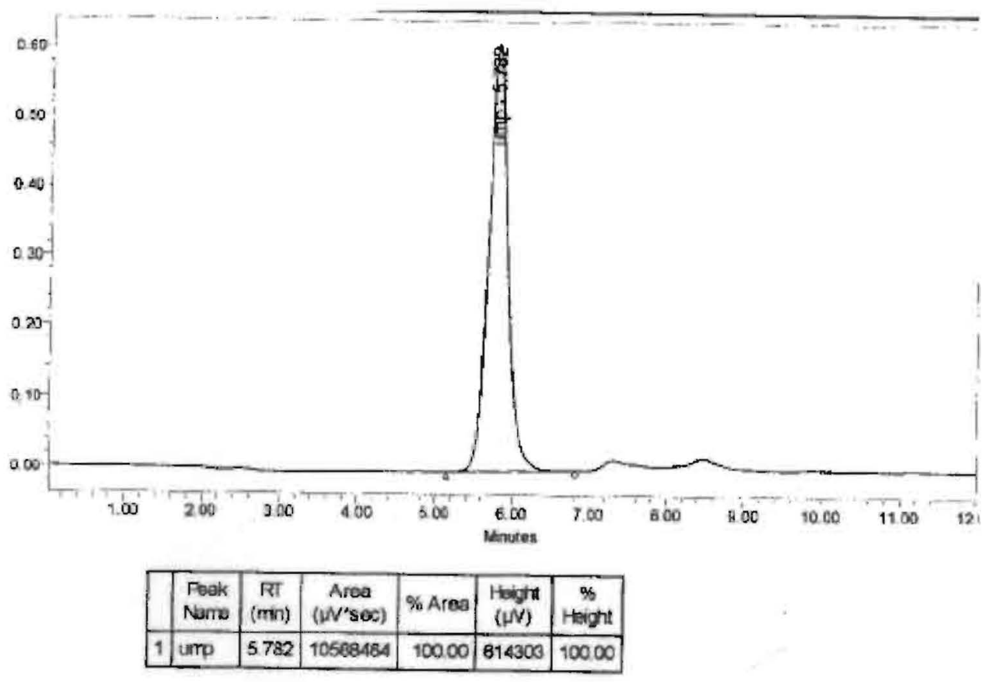
		1000 + δ
Fraction reaction (0.0 - 1.0)	0.32	
Delta value (partial rxn)	-73.087	926.913
Delta value (100% conversion)	-51.597	948.403
Isotope effect	1.0283	
User	W.O.W	

Table AI – 6: Third repeat duplicate of isotope effect calculation for natural abundance OMP reaction 3



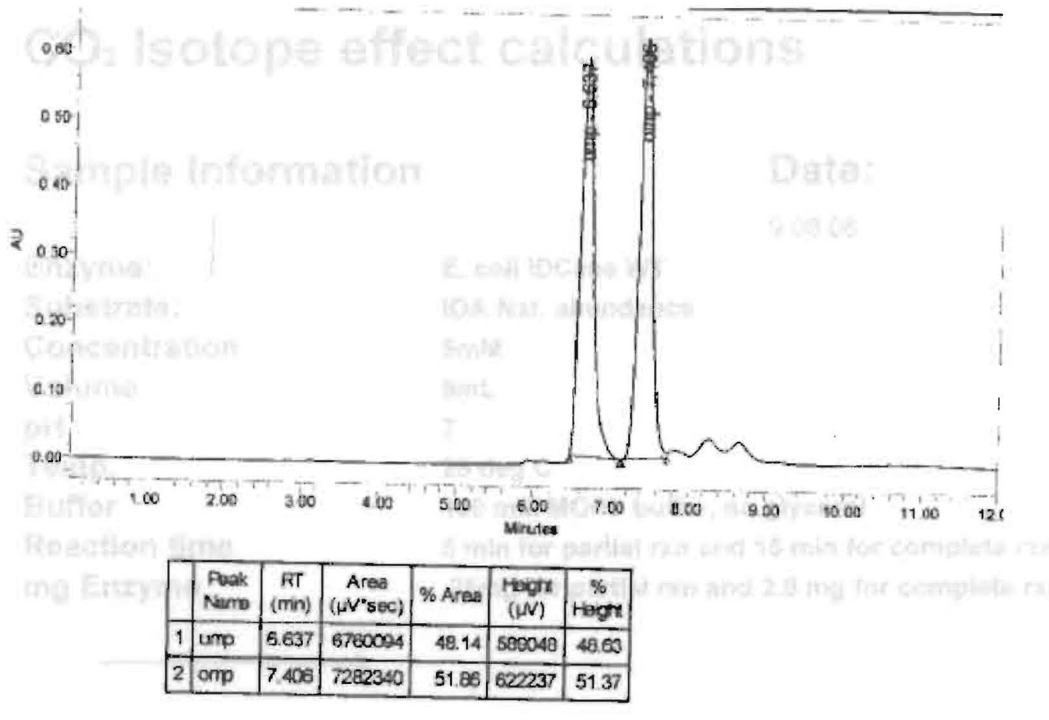
	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	?	5.937	196573	2.22	10269	2.14
2	omp	6.636	8647071	97.78	468733	97.86

Figure AI – 7: HPLC chromatogram showing unreacted OMP



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	5.782	10568464	100.00	614303	100.00

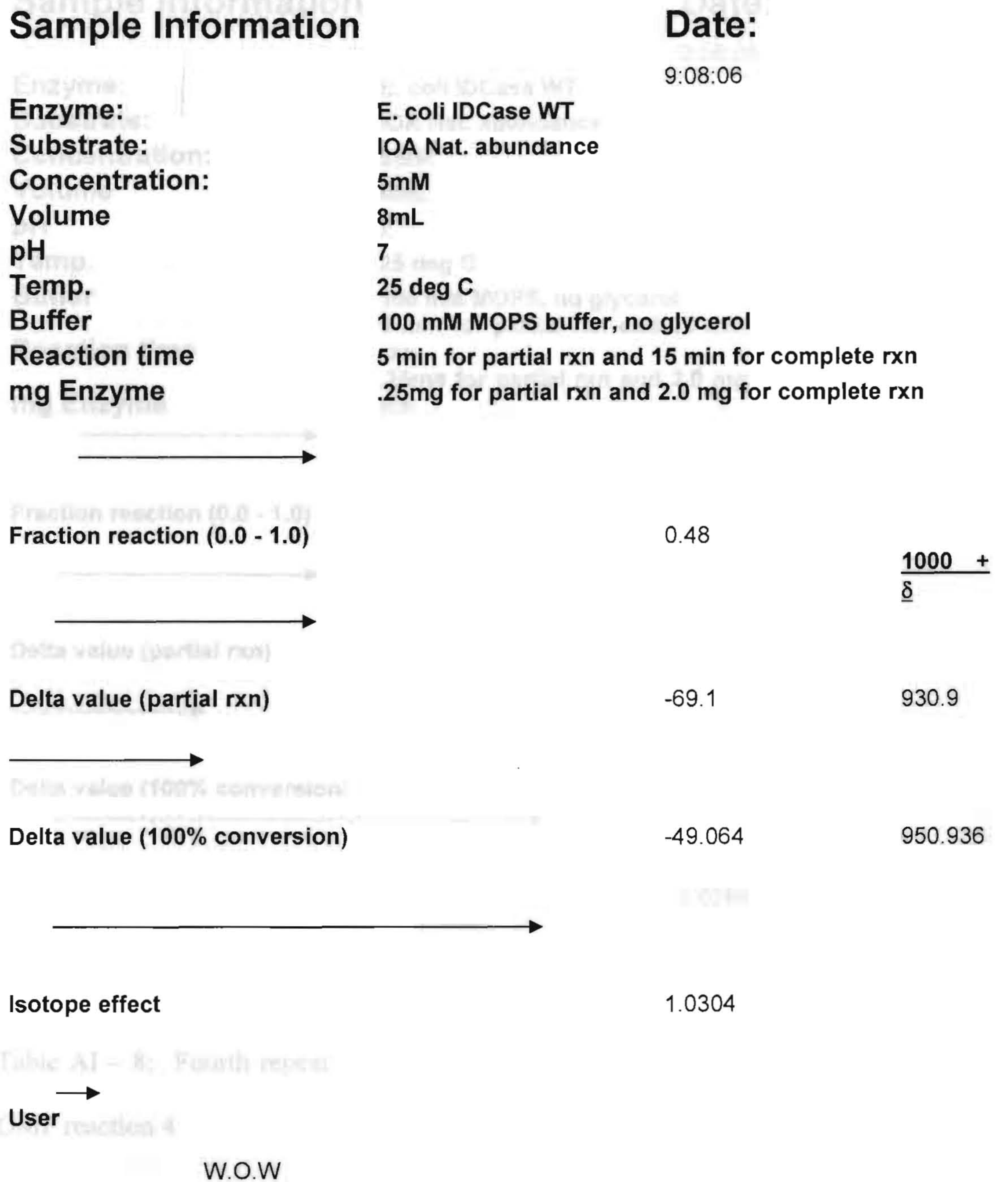
Figure AI – 8: HPLC chromatogram showing completed reaction



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	6.637	6760094	48.14	589048	48.63
2	omp	7.406	7282340	51.86	622237	51.37

Figure AI – 9: HPLC chromatogram showing partial reaction 4

CO_2 Isotope effect calculations



Sample Information

Date: 9:08:06

Enzyme:	**E. coli IDCase WT**
Substrate:	**IOA Nat. abundance**
Concentration:	**5mM**
Volume	**8mL**
pH	**7**
Temp.	**25 deg C**
Buffer	**100 mM MOPS buffer, no glycerol**
Reaction time	**5 min for partial rxn and 15 min for complete rxn**
mg Enzyme	**.25mg for partial rxn and 2.0 mg for complete rxn**

Fraction reaction (0.0 - 1.0)	0.48	**1000 + δ**
Delta value (partial rxn)	-69.1	930.9
Delta value (100% conversion)	-49.064	950.936
Isotope effect	1.0304	

User

W.O.W

Table AI – 7: Fourth repeat of isotope effect calculation for natural abundance OMP reaction 4

CO_2 Isotope effect calculations

Sample Information

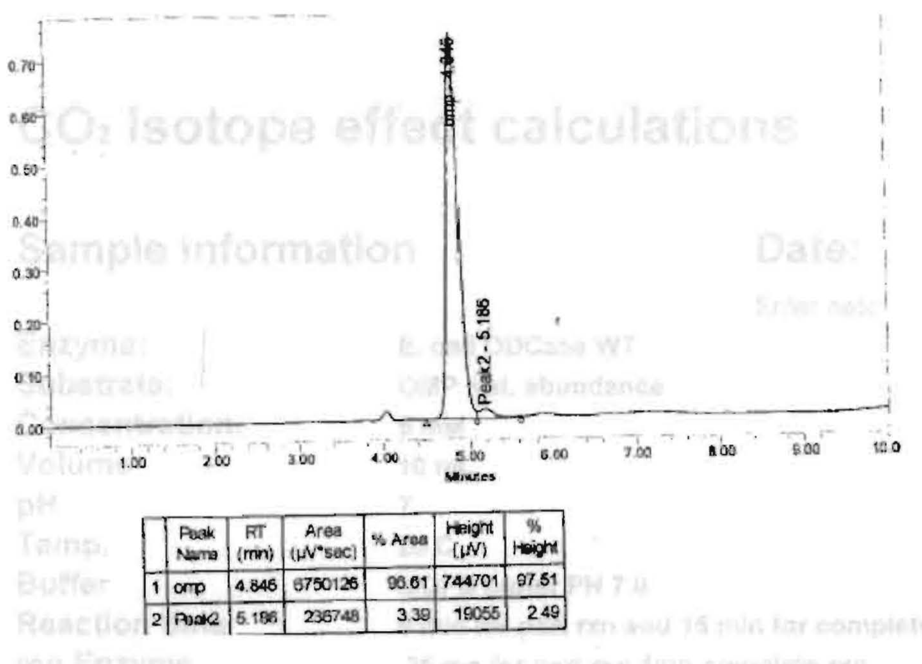
Date:

9:08:06

Enzyme:	**E. coli IDCase WT**
Substrate:	**IOA Nat. abundance**
Concentration:	**5mM**
Volume	**8mL**
pH	**7**
Temp.	**25 deg C**
Buffer	**100 mM MOPS, no glycerol**
Reaction time	**5 min for partial rxn and 15 min for complete rxn**
mg Enzyme	**.25mg for partial rxn and 2.0 mg for complete rxn**

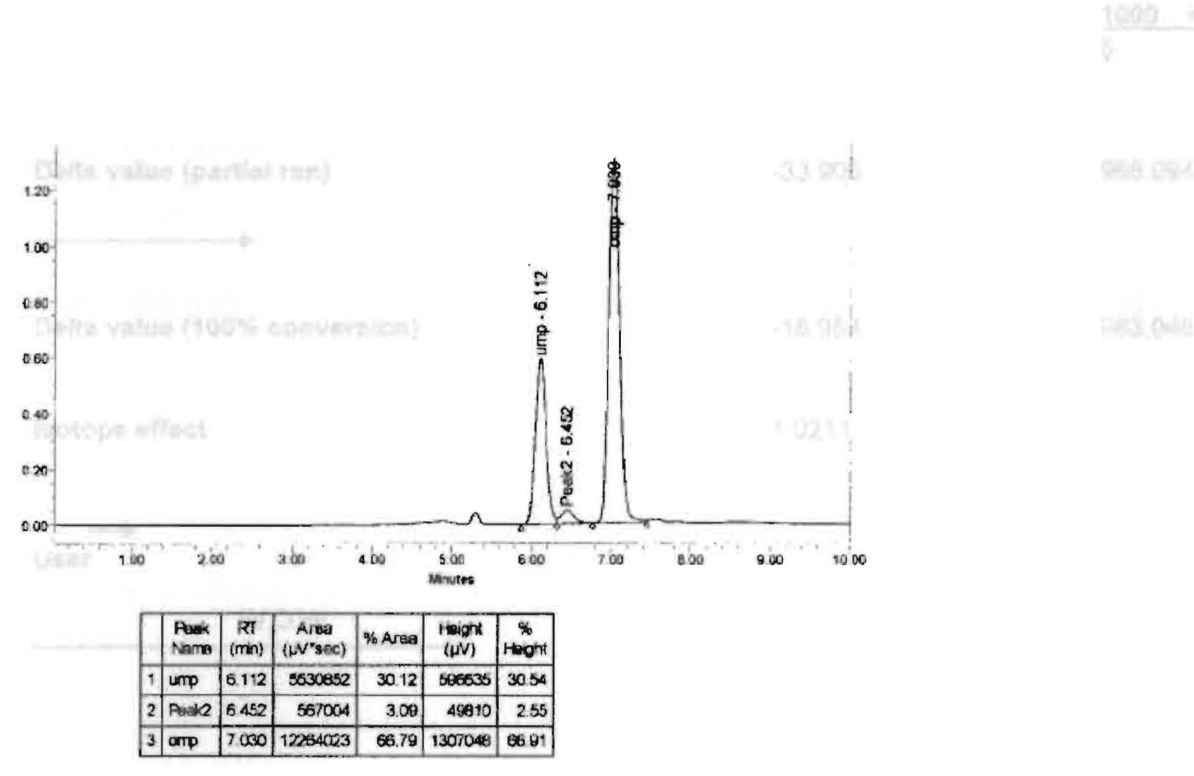
Fraction reaction (0.0 - 1.0)	0.48
Delta value (partial rxn)	-70.306
Delta value (100% conversion)	-51.428
Isotope effect	1.0286
User	W.O.W

Table AI – 8: Fourth repeat duplicate of isotope effect calculation for natural abundance OMP reaction 4



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	omp	4.846	6750126	96.61	744701	97.51
2	Peak2	5.186	236748	3.39	19055	2.49

Figure AI – 10: HPLC chromatogram showing complete reaction 1 of isotopically labeled OMP



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	6.112	5530852	30.12	596635	30.54
2	Peak2	6.452	567004	3.09	49810	2.55
3	omp	7.030	12264023	66.79	1307048	66.91

Figure AI – 11: HPLC chromatogram showing partial reaction1

CO_2 Isotope effect calculations

Sample Information

Date:

Enter date 10/23/06

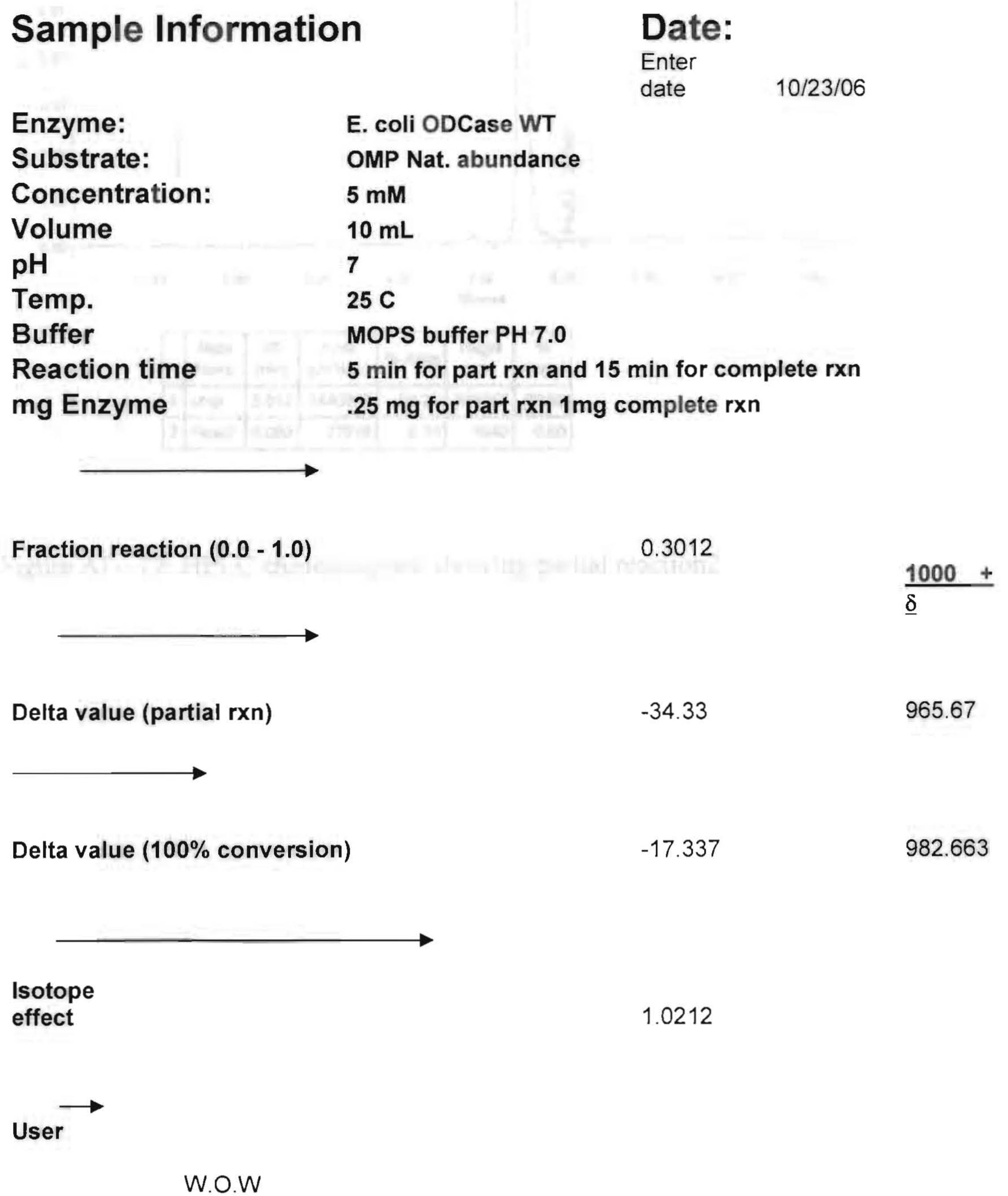
Enzyme:	**E. coli ODCase WT**
Substrate:	**OMP Nat. abundance**
Concentration:	**5 mM**
Volume	**10 mL**
pH	**7**
Temp.	**25 C**
Buffer	**MOPS buffer PH 7.0**
Reaction time	**5 min for part rxn and 15 min for complete rxn**
mg Enzyme	**.25 mg for part rxn 1mg complete rxn**

Fraction reaction (0.0 - 1.0)	0.3012	
		1000 + δ
Delta value (partial rxn)	-33.906	966.094
Delta value (100% conversion)	-16.954	983.046
Isotope effect	1.0211	

User

W.O.W

Table AI – 9: Isotope effect calculation for isotopically labeled OMP reaction 1



CO_2 Isotope effect calculations

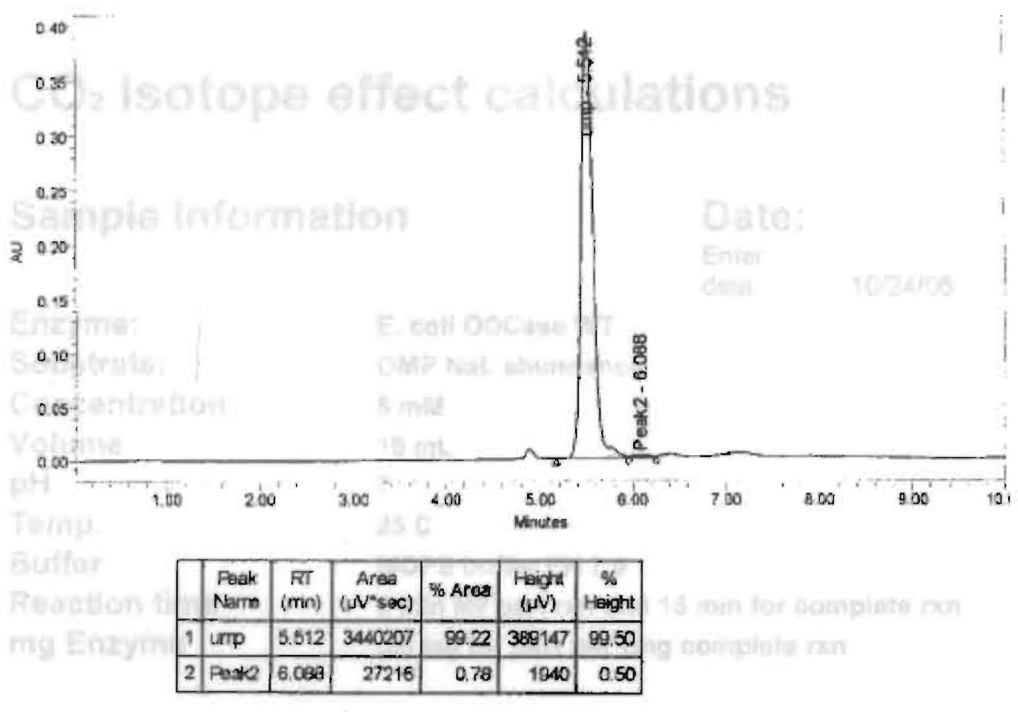
Sample Information

Date:
Enter date 10/23/06

Enzyme:	E. coli ODCase WT
Substrate:	OMP Nat. abundance
Concentration:	5 mM
Volume	10 mL
pH	7
Temp.	25 C
Buffer	MOPS buffer PH 7.0
Reaction time	5 min for part rxn and 15 min for complete rxn
mg Enzyme	.25 mg for part rxn 1mg complete rxn

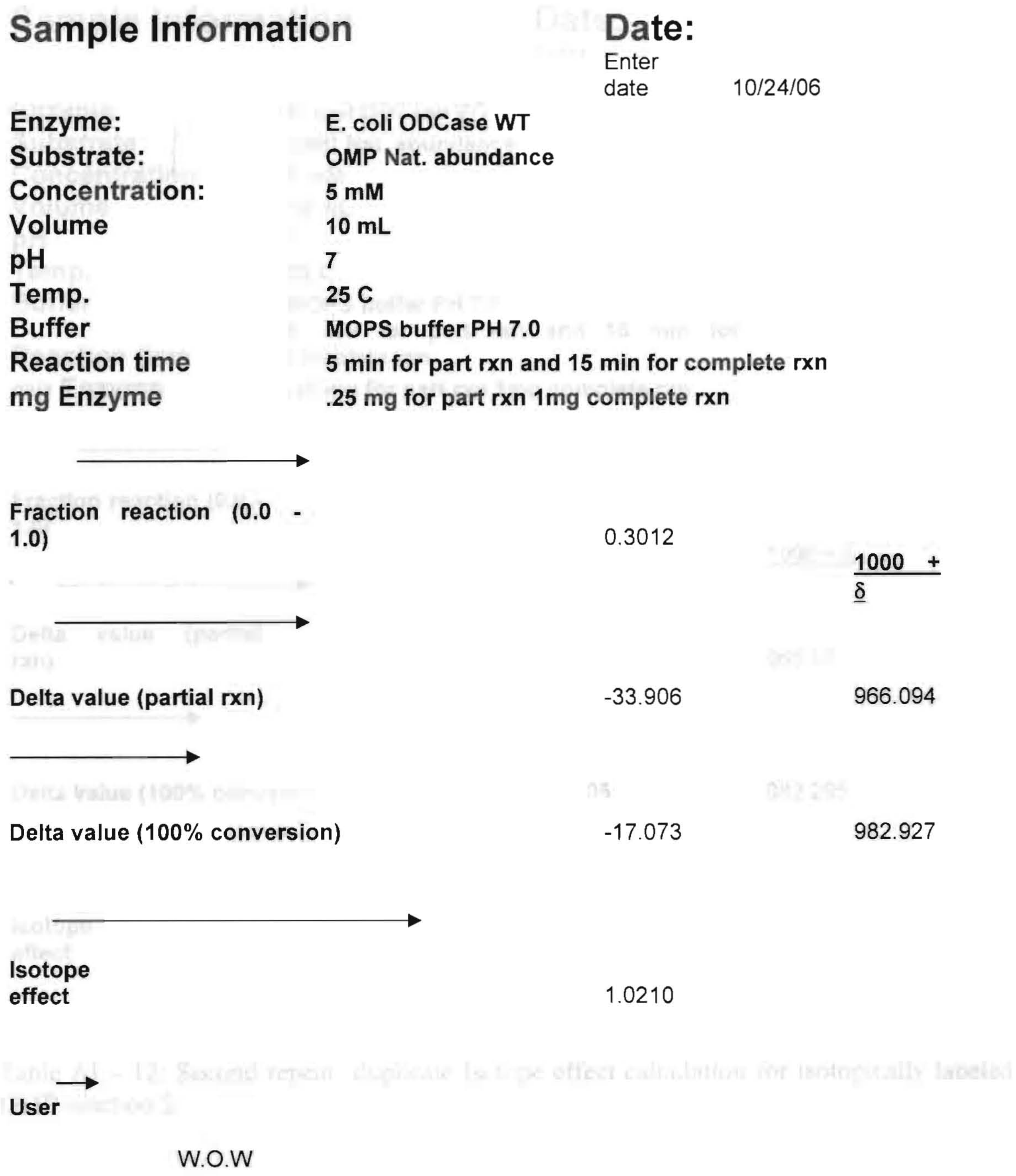
		1000 + δ
Fraction reaction (0.0 - 1.0)	0.3012	
Delta value (partial rxn)	-34.33	965.67
Delta value (100% conversion)	-17.337	982.663
Isotope effect	1.0212	
User	W.O.W	

Table AI – 10: First duplicate of isotope effect calculation for isotopically labeled OMP reaction 1



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	5.512	3440207	99.22	389147	99.50
2	Peak2	6.088	27216	0.78	1940	0.50

Figure AI – 12: HPLC chromatogram showing partial reaction2



CO_2 Isotope effect calculations

Sample Information

Date:

Enter date 10/24/06

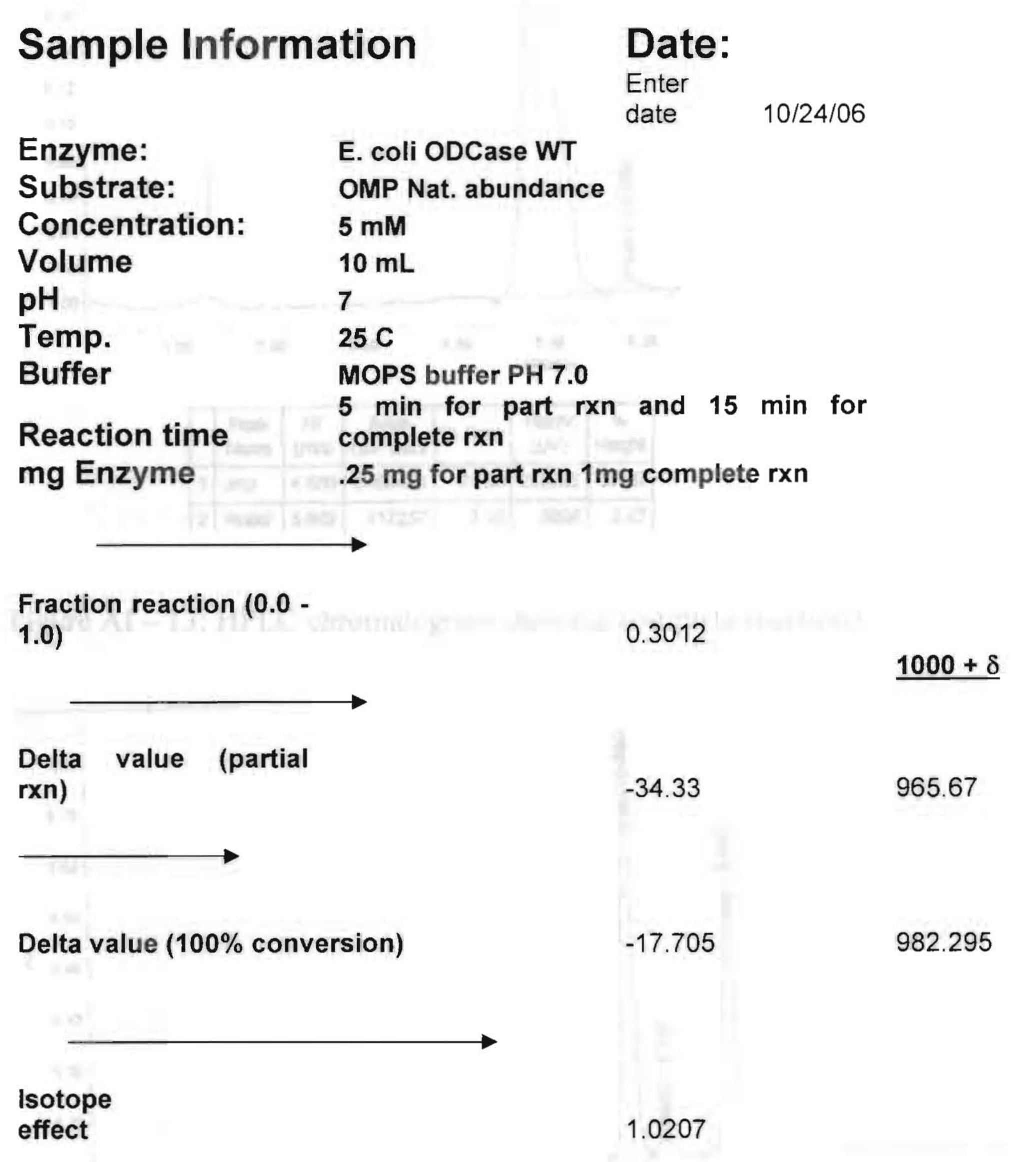
Enzyme:	**E. coli ODCase WT**
Substrate:	**OMP Nat. abundance**
Concentration:	**5 mM**
Volume	**10 mL**
pH	**7**
Temp.	**25 C**
Buffer	**MOPS buffer PH 7.0**
Reaction time	**5 min for part rxn and 15 min for complete rxn**
mg Enzyme	**.25 mg for part rxn 1mg complete rxn**

		$1000 + \delta$
Fraction reaction (0.0 - 1.0)	0.3012	
Delta value (partial rxn)	-33.906	966.094
Delta value (100% conversion)	-17.073	982.927
Isotope effect	1.0210	

User

W.O.W

Table AI – 11: Second repeat Isotope effect calculation for isotopically labeled OMP reaction2



CO_2 Isotope effect calculations

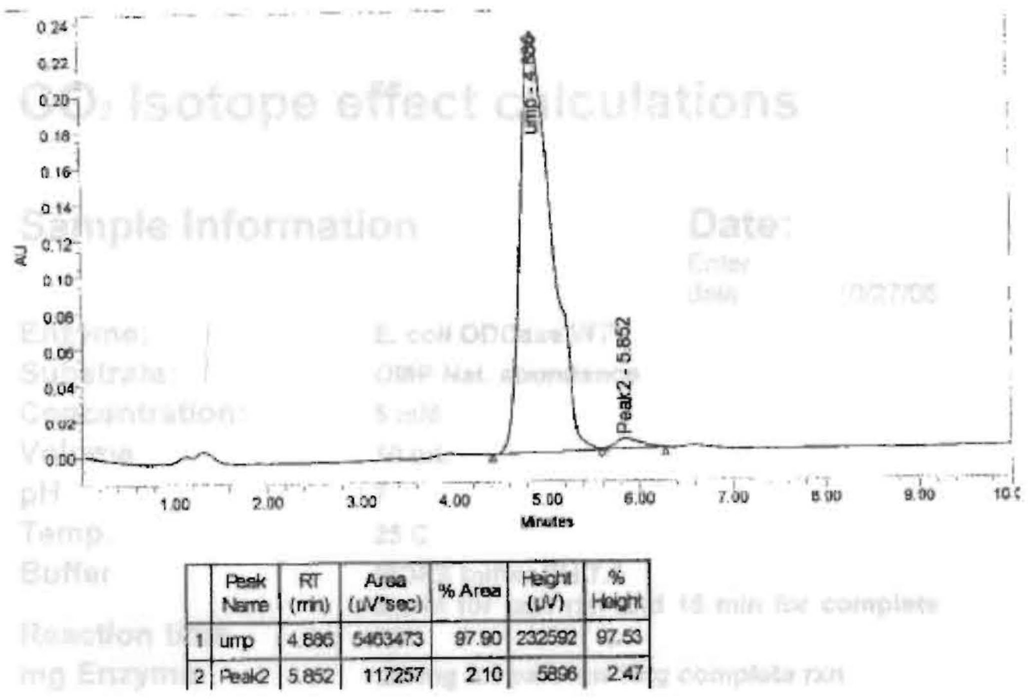
Sample Information

Date:
Enter date 10/24/06

Enzyme:	**E. coli ODCase WT**
Substrate:	**OMP Nat. abundance**
Concentration:	**5 mM**
Volume	**10 mL**
pH	**7**
Temp.	**25 C**
Buffer	**MOPS buffer PH 7.0**
Reaction time	**5 min for part rxn and 15 min for complete rxn**
mg Enzyme	**.25 mg for part rxn 1mg complete rxn**

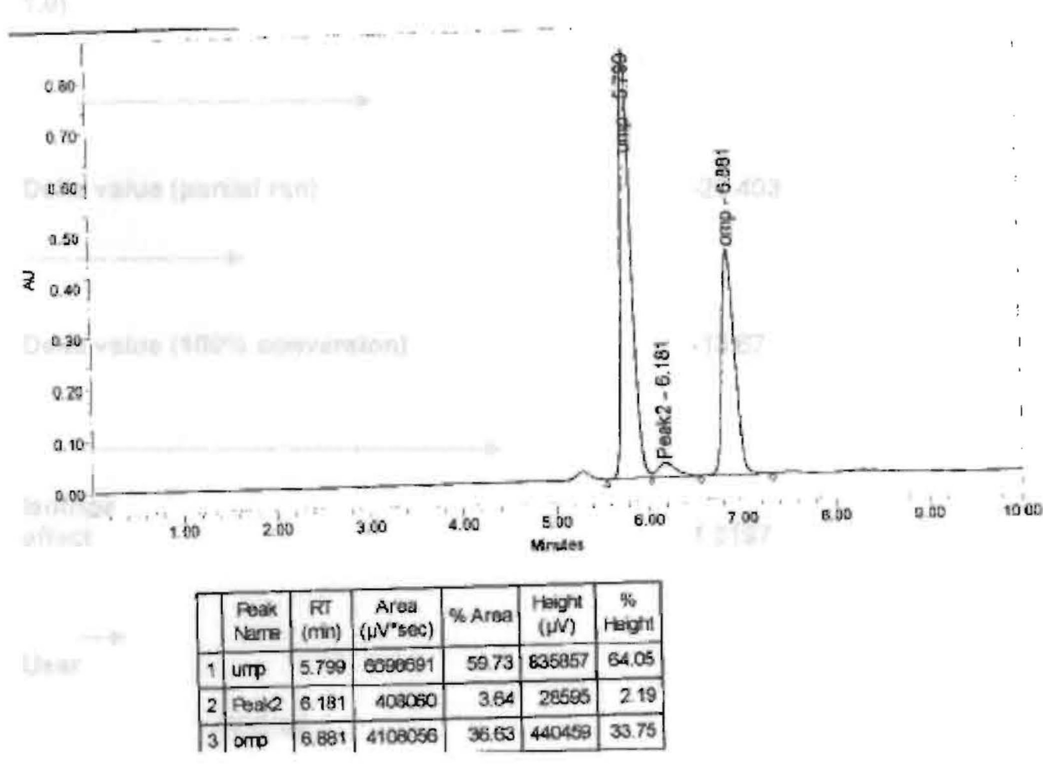
		$1000 + \delta$
Fraction reaction (0.0 - 1.0)	0.3012	
Delta value (partial rxn)	-34.33	965.67
Delta value (100% conversion)	-17.705	982.295
Isotope effect	1.0207	

Table AI – 12: Second repeat duplicate Isotope effect calculation for isotopically labeled OMP reaction 2



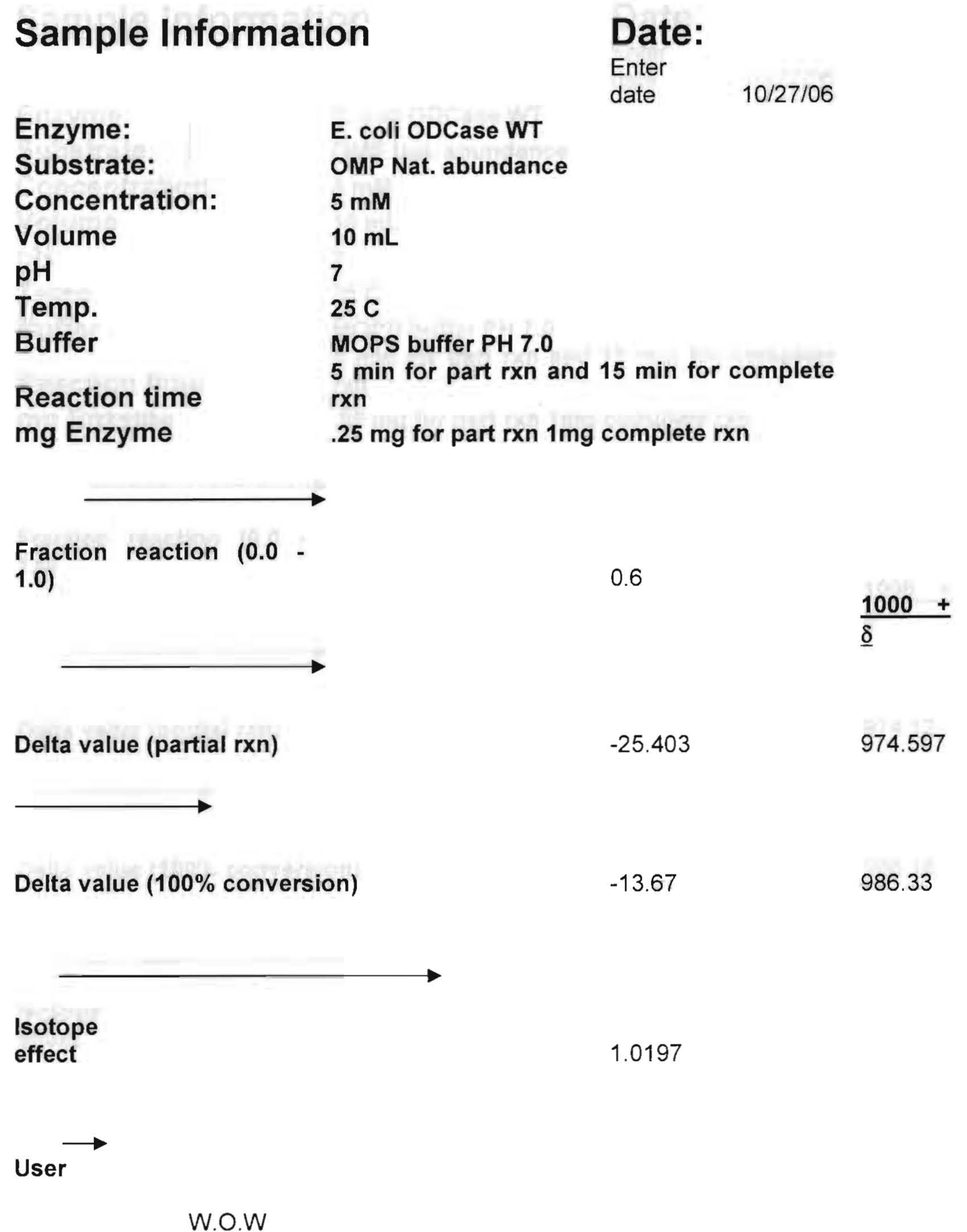
	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	4.886	5463473	97.90	232592	97.53
2	Peak2	5.852	117257	2.10	5896	2.47

Figure AI – 13: HPLC chromatogram showing complete reaction3



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	5.799	6698691	59.73	835857	64.05
2	Peak2	6.181	408060	3.64	28595	2.19
3	omp	6.881	4108056	36.63	440459	33.75

Figure AI – 14: HPLC chromatogram showing partial reaction3



CO_2 Isotope effect calculations

Sample Information

Date:
Enter date 10/27/06

Enzyme:	**E. coli ODCase WT**
Substrate:	**OMP Nat. abundance**
Concentration:	**5 mM**
Volume	**10 mL**
pH	**7**
Temp.	**25 C**
Buffer	**MOPS buffer PH 7.0**
Reaction time	**5 min for part rxn and 15 min for complete rxn**
mg Enzyme	**.25 mg for part rxn 1mg complete rxn**

		1000 + δ
Fraction reaction (0.0 - 1.0)	0.6	
Delta value (partial rxn)	-25.403	974.597
Delta value (100% conversion)	-13.67	986.33
Isotope effect	1.0197	

User

W.O.W

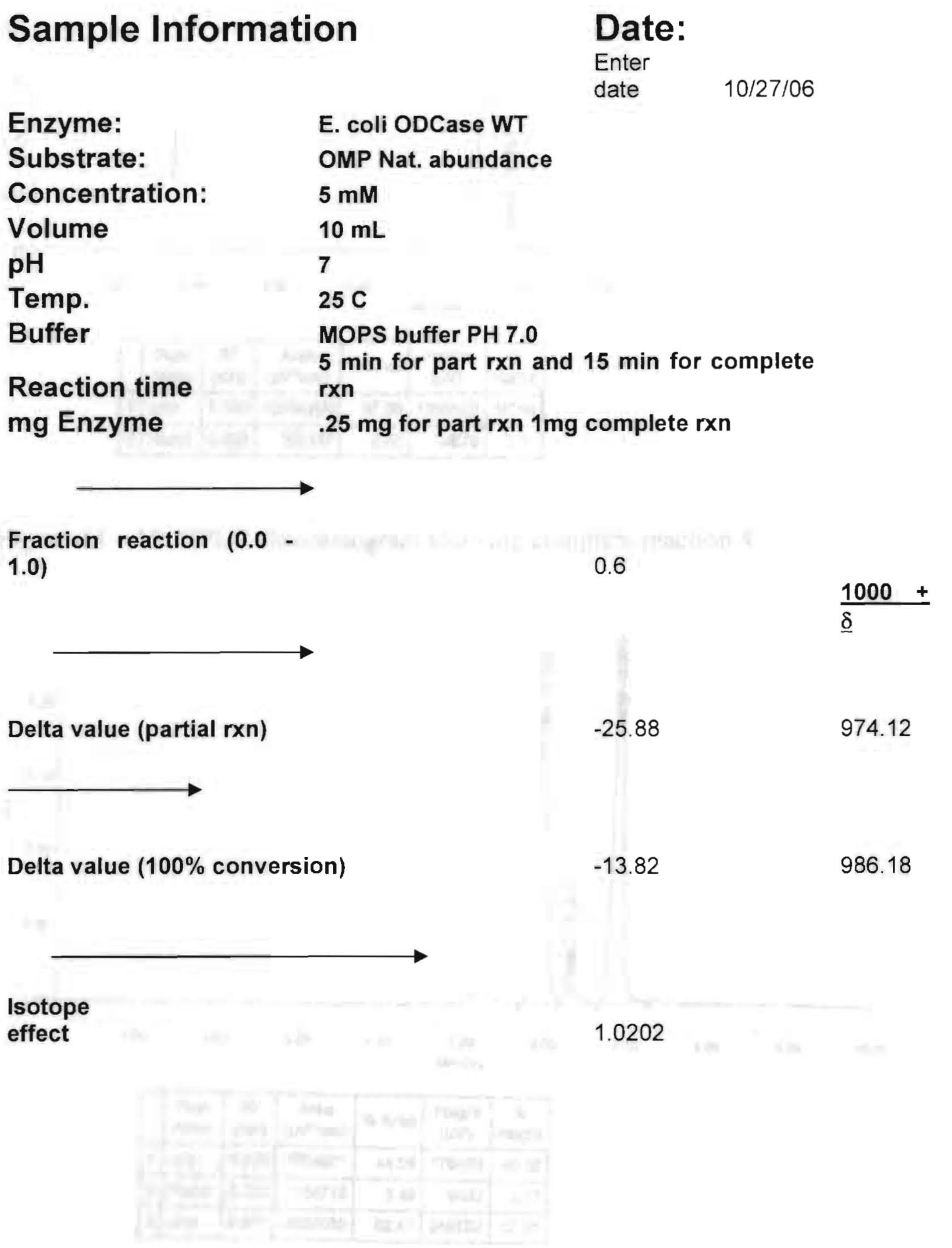
Table AI – 13: Third repeat Isotope effect calculation for isotopically labeled OMP reaction

CO_2 Isotope effect calculations

Sample Information

Date:

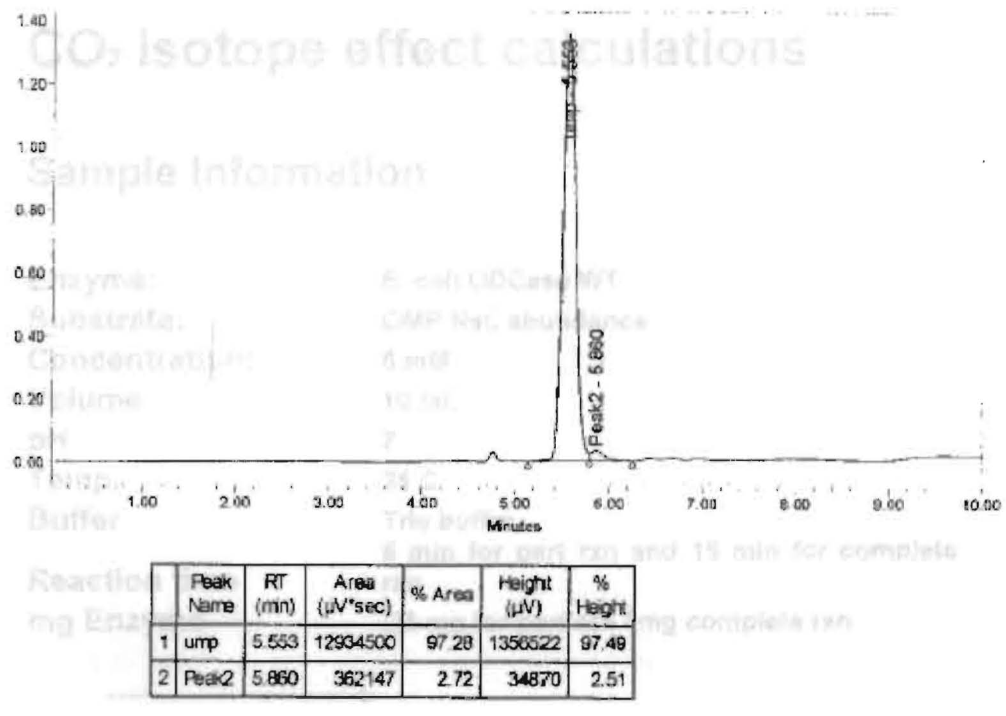
Enter date 10/27/06



Enzyme:	**E. coli ODCase WT**
Substrate:	**OMP Nat. abundance**
Concentration:	**5 mM**
Volume	**10 mL**
pH	**7**
Temp.	**25 C**
Buffer	**MOPS buffer PH 7.0**
Reaction time	**5 min for part rxn and 15 min for complete rxn**
mg Enzyme	**.25 mg for part rxn 1mg complete rxn**

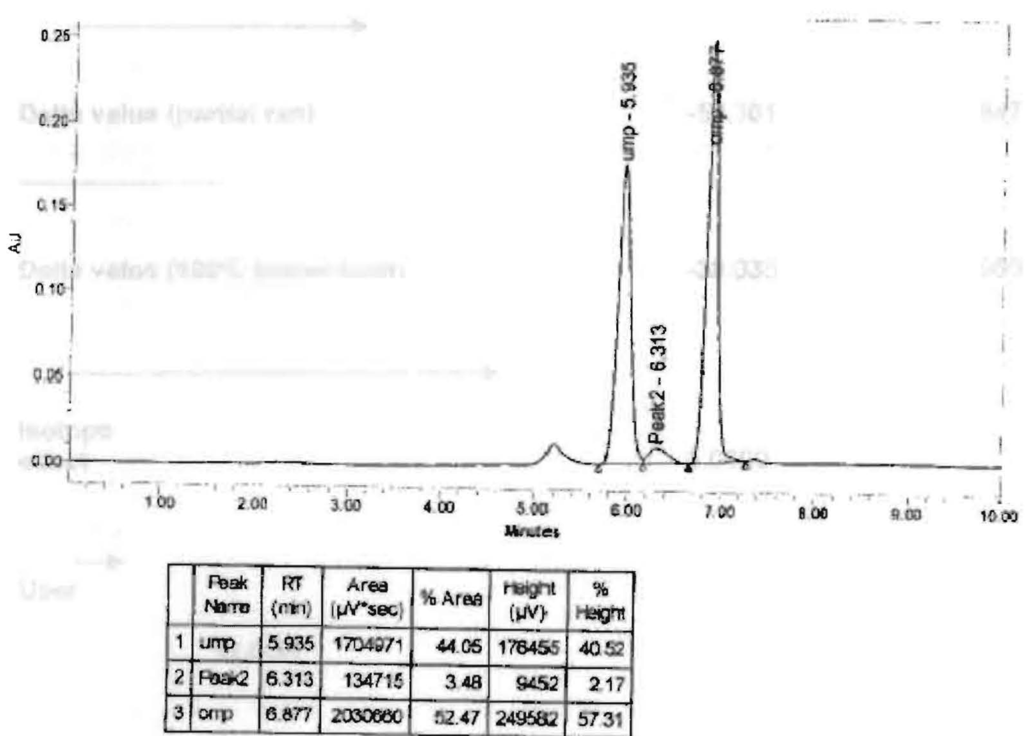
Fraction reaction (0.0 - 1.0) →	0.6	<u>1000 +</u> <u>δ</u>
Delta value (partial rxn) →	-25.88	974.12
Delta value (100% conversion) →	-13.82	986.18
Isotope effect →	1.0202	

Table AI – 14: Third repeat duplicate Isotope effect calculation for isotopically labeled OMP reaction 3



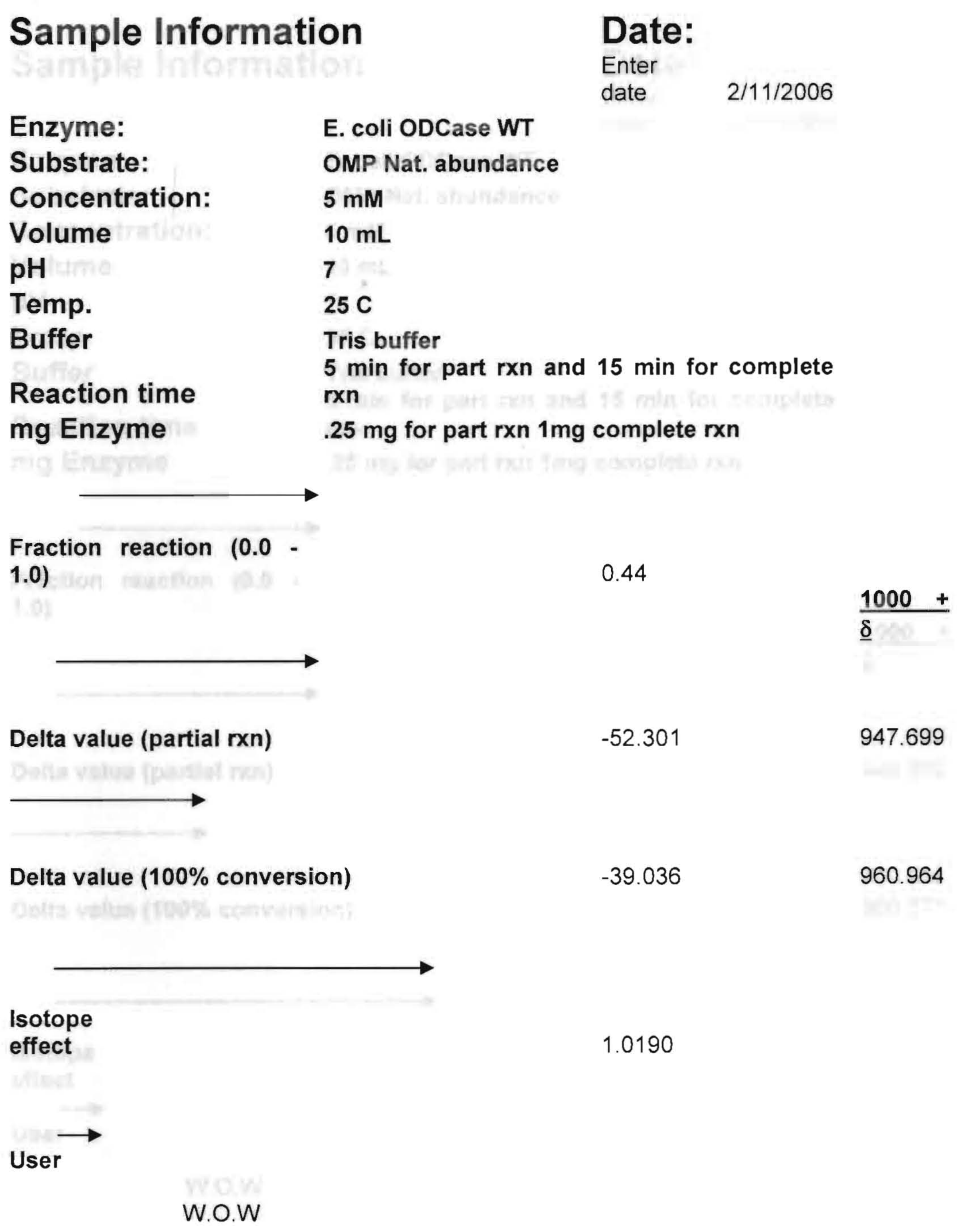
	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	5.553	12934500	97.28	1356522	97.49
2	Peak2	5.860	362147	2.72	34870	2.51

Figure AI – 15: HPLC chromatogram showing complete reaction 4



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	5.935	1704971	44.05	176455	40.52
2	Peak2	6.313	134715	3.48	9452	2.17
3	omp	6.877	2030660	52.47	249582	57.31

Figure AI – 16: HPLC chromatogram showing partial reaction 4



CO_2 Isotope effect calculations

Sample Information

Date:
Enter date 2/11/2006

Enzyme:	**E. coli ODCase WT**
Substrate:	**OMP Nat. abundance**
Concentration:	**5 mM**
Volume	**10 mL**
pH	**7**
Temp.	**25 C**
Buffer	**Tris buffer**
Reaction time	**5 min for part rxn and 15 min for complete rxn**
mg Enzyme	**.25 mg for part rxn 1mg complete rxn**

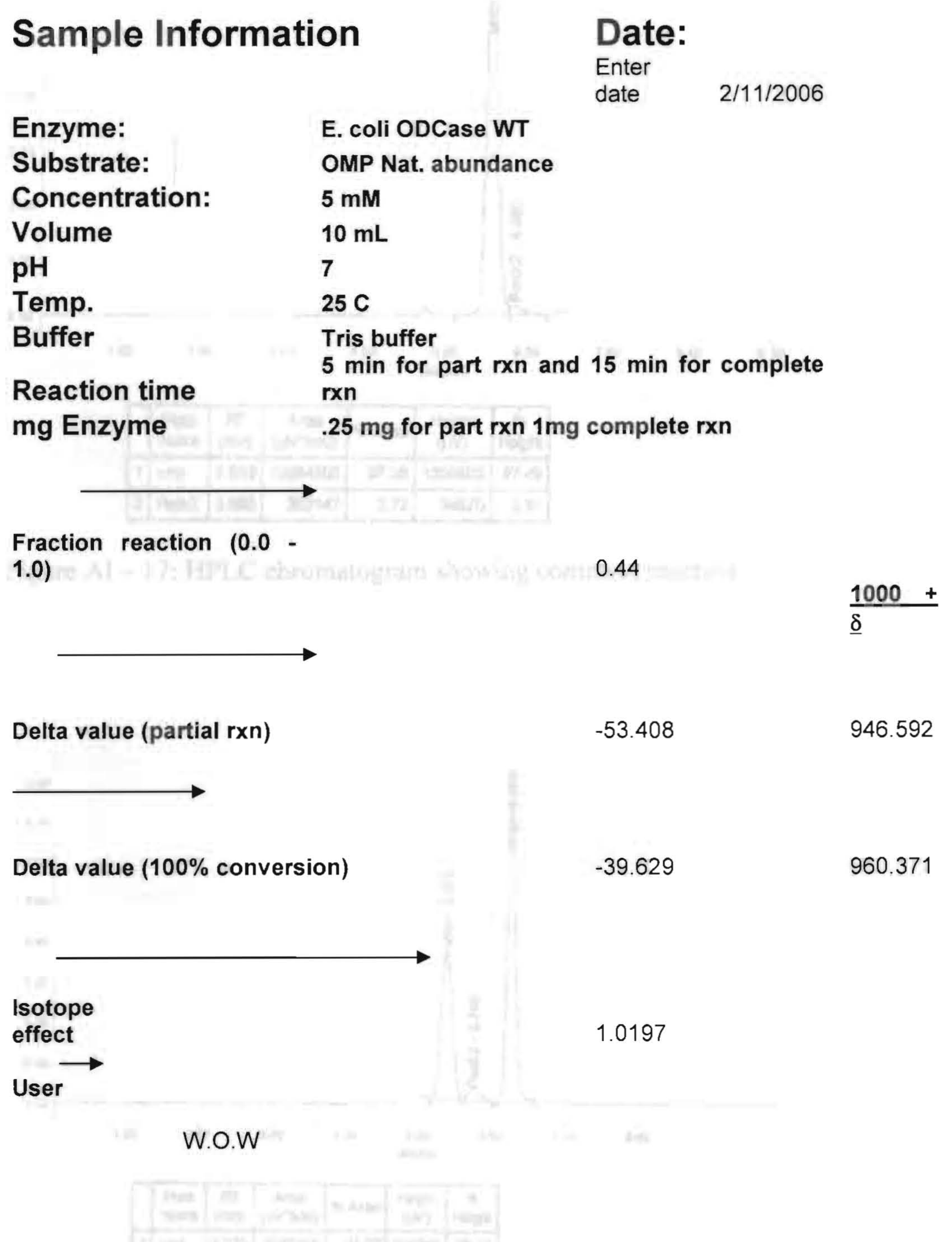
		1000 + δ
Fraction reaction (0.0 - 1.0)	0.44	
Delta value (partial rxn)	-52.301	947.699
Delta value (100% conversion)	-39.036	960.964
Isotope effect	1.0190	

User

W.O.W

Table AI – 15: Fourth repeat Isotope effect calculation for isotopically labeled OMP reaction 4

CO_2 Isotope effect calculations



Sample Information

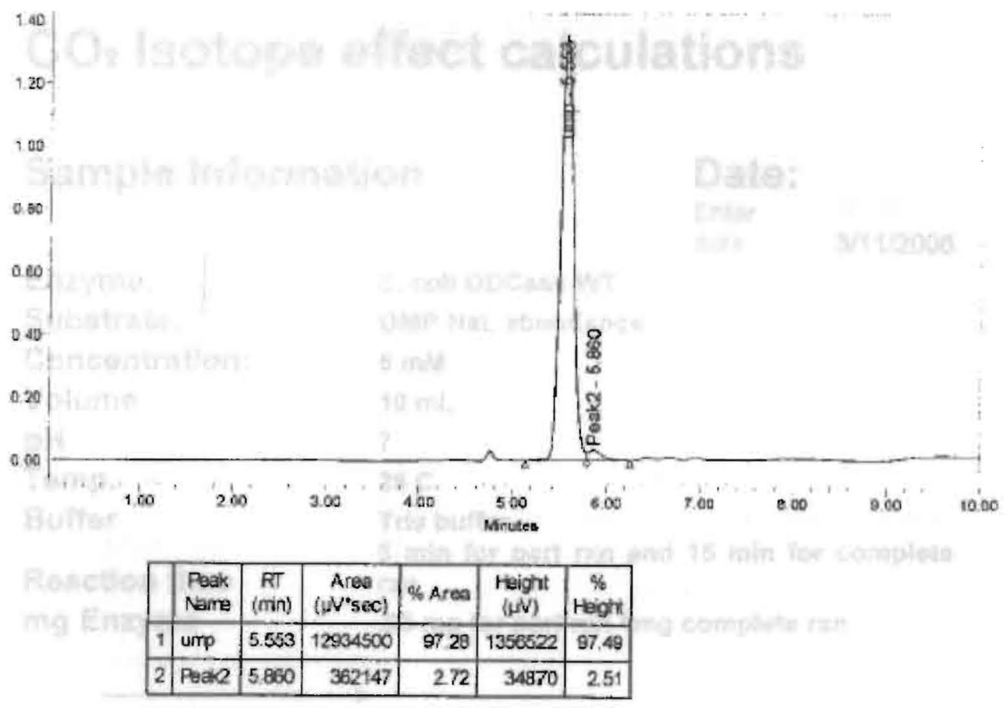
Date:
Enter date 2/11/2006

Enzyme:	**E. coli ODCase WT**
Substrate:	**OMP Nat. abundance**
Concentration:	**5 mM**
Volume	**10 mL**
pH	**7**
Temp.	**25 C**
Buffer	**Tris buffer**
Reaction time	**5 min for part rxn and 15 min for complete rxn**
mg Enzyme	**.25 mg for part rxn 1mg complete rxn**

		$\frac{1000+}{\delta}$
Fraction reaction (0.0 - 1.0) →	0.44	
Delta value (partial rxn) →	-53.408	946.592
Delta value (100% conversion) →	-39.629	960.371
Isotope effect →	1.0197	
User		

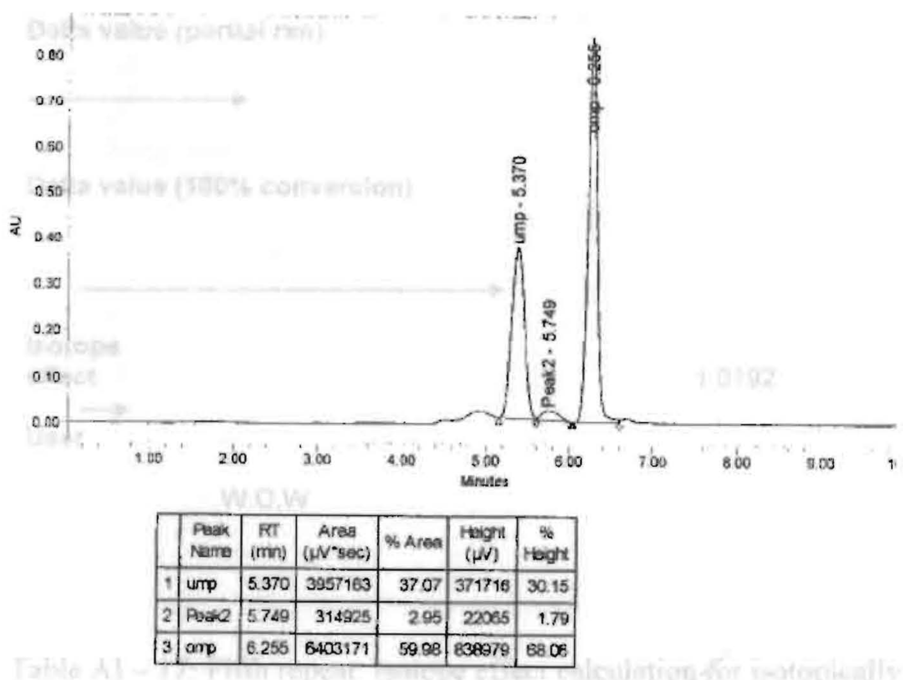
W.O.W

Table AI – 16: Fourth repeat duplicate Isotope effect calculation for isotopically labeled OMP reaction



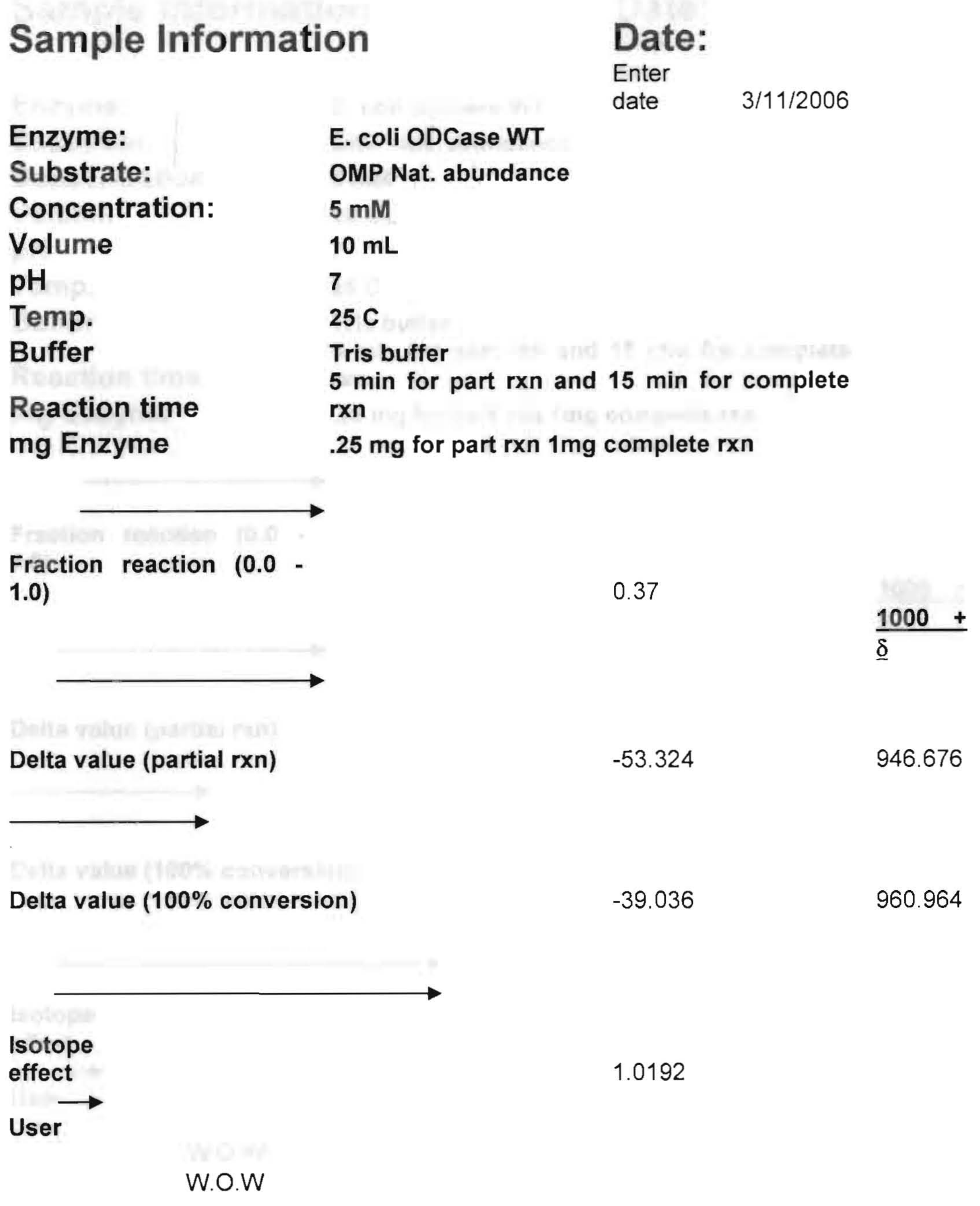
	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	5.553	12934500	97.28	1356522	97.49
2	Peak2	5.860	362147	2.72	34870	2.51

Figure AI – 17: HPLC chromatogram showing complete reaction 4



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	ump	5.370	3957163	37.07	371716	30.15
2	Peak2	5.749	314925	2.95	22065	1.79
3	omp	6.255	6403171	59.98	838979	68.06

Figure AI – 18: HPLC chromatogram showing partial reaction 4



CO_2 Isotope effect calculations

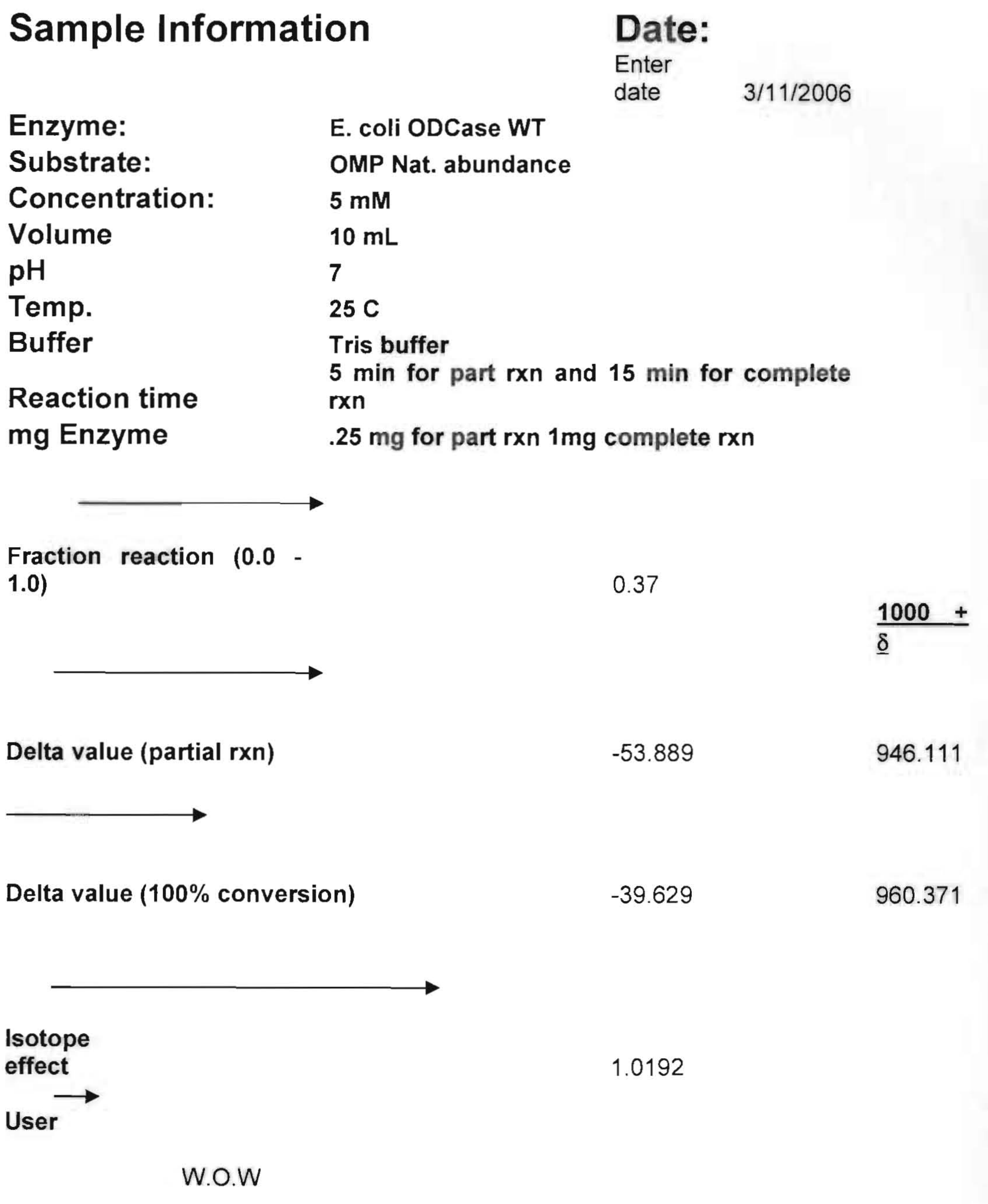
Sample Information

Date:
Enter date 3/11/2006

Enzyme:	**E. coli ODCase WT**
Substrate:	**OMP Nat. abundance**
Concentration:	**5 mM**
Volume	**10 mL**
pH	**7**
Temp.	**25 C**
Buffer	**Tris buffer**
Reaction time	**5 min for part rxn and 15 min for complete rxn**
mg Enzyme	**.25 mg for part rxn 1mg complete rxn**

		$1000 + \delta$
Fraction reaction (0.0 - 1.0)	0.37	
Delta value (partial rxn)	-53.324	946.676
Delta value (100% conversion)	-39.036	960.964
Isotope effect	1.0192	
User	W.O.W	

Table AI – 17: Fifth repeat Isotope effect calculation for isotopically labeled OMP reaction 5



CO_2 Isotope effect calculations

Sample Information

Date:
Enter date 3/11/2006

Enzyme:	E. coli ODCase WT
Substrate:	OMP Nat. abundance
Concentration:	5 mM
Volume	10 mL
pH	7
Temp.	25 C
Buffer	Tris buffer
Reaction time	5 min for part rxn and 15 min for complete rxn
mg Enzyme	.25 mg for part rxn 1mg complete rxn

Fraction reaction (0.0 - 1.0)	0.37	
		1000 + δ
Delta value (partial rxn)	-53.889	946.111
Delta value (100% conversion)	-39.629	960.371
Isotope effect	1.0192	
User	W.O.W	

Table AI – 18: Fifth repeat duplicate Isotope effect calculation for isotopically labeled OMP reaction 5