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Paracetamol – an inhibitor of Sorbitol dehydrogenase?

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Abstract

Paracetamol is one of the most common used analgesics and antipyretics in the world. Still, its exact mechanisms of action are unknown.

Sorbitol dehydrogenase is an enzyme that catalyze the oxidation of the polyhydric alcohol D-sorbitol to D-fructose. Sorbitol dehydrogenase uses NAD^+/ NADH as a coenzyme.

The work of this thesis has been a research on whether paracetamol can be an inhibitor of sorbitol dehydrogenase, and eventually what kind of inhibition it would be, and whether it binds to the active site. AutoDock Vina was to be used to verify the results from laboratory work.

Experimental work has been done in the laboratory, using spectrophotometric methods to find the maximum rate, V_{max}, and the affinity value K_M of the reaction where D-sorbitol is oxidized to D-fructose, both in the presence of paracetamol and without, at pH 9.9.

Four series of 10 reaction samples were made, where the series had an increasing paracetamol concentration, and the 10 reaction samples of each series had increasing D-sorbitol concentration. In addition to D-sorbitol and paracetamol, each reaction sample contained 50mM NAD^+ and glycine buffer (pH 9.9).

The initial reaction rate for each of the reaction solutions was estimated by measuring the change in absorbance by NADH at 340nm over a period of ten seconds, shortly after the enzyme was added to the reaction solution. A Lineweaver-Burk plot was used to find V_{max} and K_M for each of the four series, and to see whether paracetamol influences V_{max} and K_M for the reaction.

The results indicate that paracetamol is indeed an inhibitor of sorbitol dehydrogenase. The results from the laboratory work show that both the maximum velocity of the reaction, as well as the enzyme's affinity for the substrate D-sorbitol changes in the presence of paracetamol.

The results from the docking in AutoDock Vina verifies that paracetamol has a great binding affinity to the active site of sorbitol dehydrogenase, where it binds, along with the coenzyme NAD^+/ NADH, towards the catalytic zinc atom.

It is not clear given the results what kind of inhibition the paracetamol present to the sorbitol dehydrogenase. However, they might indicate that there is a form of mixed inhibition.

Sammendrag

Paracetamol er et av de mest brukte analgetika i verden. Likevel er dens eksakte funksjonsmekanisme ukjent.

Sorbitol dehydrogenase er et enzym som katalyserer oksidasjonen av sukkeralkoholen D-sorbitol til D-fruktose. Sorbitol dehydrogenase bruker NAD+/NADH som koenzym.

Arbeidet i denne masteren har vært en studie på om paracetamol kan være en hemmer av sorbitol dehydrogenase, og eventuelt hva slags hemming det ville være, og om den binder til det aktive setet. AutoDock Vina er blitt brukt til å bekrefte resultater fra laboratoriearbeidet.

Eksperimentelt arbeid er gjort på laboratorium, der spektrofotometriske metoder er blitt brukt til å finne V_{max} og K_M til reaksjonen der D-sorbitol blir oksidert til D-fruktose, både med paracetamol tilstede og uten, ved pH 9.9.

Fire serier med 10 reaksjonsprøver ble laget, der seriene hadde økende paracetamol konsentrasjon, og de 10 reaksjonsprøvene i hver serie hadde økende D-sorbitol konsentrasjon. I tillegg til D-sorbitol og paracetamol inneholdt hver reaksjonsprøve av 50mM NAD^+ og glycin buffer (pH 9.9).

Den initiale reaksjonshastigheten for hver av reaksjonsløsningene ble estimert ved å måle endringen i absorbans av NADH ved 340nm over ti sekunder, kort tid etter at enzymet sorbitol dehydrogenase ble tilsatt reaksjonsblandingen. Lineweaver-Burk plott ble brukt til å finne V_{max} og K_M for hver av de fire seriene, og for å se om paracetamol påvirker V_{max} og K_M for reaksjonen.

Resultatene indikerer at paracetamol er en hemmer av sorbitol dehydrogenase. Resultatene fra laboratoriearbeidet viser at både den maksimale reaksjonshastigheten og enzymets affinitet for substratet D-sorbitol påvirkes av tilstedeværelsen av paracetamol.

Resultatene fra dockingen i AutoDock Vina bekrefter at paracetamol har en god bindingsaffinitet til det aktive setet til sorbitol dehydrogenase, der den binder, sammen med NAD^+/NADH, mot det katalytiske sink-atomet.

Det er ikke klart ut fra resultatene hvordan paracetamol hemmer D-sorbitol dehydrogenase. Likevel kan de tyde på at det er en form for «mixed» hemming.

Acknowledgements

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I would like to express my gratitude to you, Lars Skjeldal, for trusting me with this master thesis, and for the guidance and patience you have bestowed upon me these last months. You have supported me both professionally and morally, and without your encouraging words I am not sure how this would have ended.

I also want to thank Jonas Svåsand, my fellow student, for his help on the computational matters. I am no computer expert, and so I am endlessly grateful that you took the time to guide me through the steps of how to use the AutoDock Tools and AutoDock Vina, and that you gave me helpful advices on how to make good pictures in PyMOL.

I would also like to thank my family and friends for their encouragements. Especially I want to thank Christiane and my mom, for mental support, and my husband Magnus, whose love and patience has been crucial.

Aim

The aim of this thesis has been to see whether paracetamol can be an inhibitor of the enzyme sorbitol dehydrogenase, and what kind of inhibition this could be. Furthermore, to find out whether paracetamol binds to the active site, using computational docking to verify kinetic data collected in laboratory work.

Abbreviations

NMR	Nuclear Magnetic Resonance
NMBU	Norwegian University of Life Science
SDH	Sorbitol dehydrogenase
UV	Ultraviolet
NaOH	Sodium hydroxide
$NaH_2PO_4 \times 2H_2O$	Sodium phosphate monobasic dihydrate
$Na_2HPO_4 \times 2H_2O$	Disodium phosphate dibasic dihydrate
k	kilo (10^3)
k_{cat}	turnover number
K_M	Michaelis constant
L	liter
M	molar (mol/L)
m	milli (10^{-3})
mL	milliliter
mM	millimolar (mmol/L)
mol	mole
mmol	millimole
μ	micro (10^{-6})
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
P	phosphate
V_m	maximal velocity
nm	nano meter

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1   Introduction

1.1   History and properties of paracetamol

Paracetamol is the most commonly used analgesic and antipyretic in the western world (e.g. USA and Europe). It is considered harmless if consumed within the recommended doses, although its exact mechanisms of action remain uncertain [1]. The structure of paracetamol is shown in figure 1.1 (A and B).

It has been proposed that paracetamol has the ability to mildly inhibit the enzyme cyclooxygenase (COX) 1 and 2, and inhibits the action of COX 3 [1]. COX is involved in the making of some prostaglandins. Prostaglandins can, among other things, act as a mediating agent in the process of inflammation, fever and allergy. Some can also promote pain, swelling and redness, by sensitizing pain receptors and increasing blood flow [2].

The most common injury following from an overdose of paracetamol is acute liver failure. It is suggested that this is the consequence of the excess production of the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). NAPQI is one of three main metabolites of paracetamol, but only 5 % of the absorbed paracetamol will be synthesized into NAPQI, and this will not do any harm. If, however, paracetamol is consumed in larger amounts, NAPQI will be able to bind cellular thiol groups, and thereby cause cell death. The structure of NAPQI is shown in figure 1.2 (A and B).
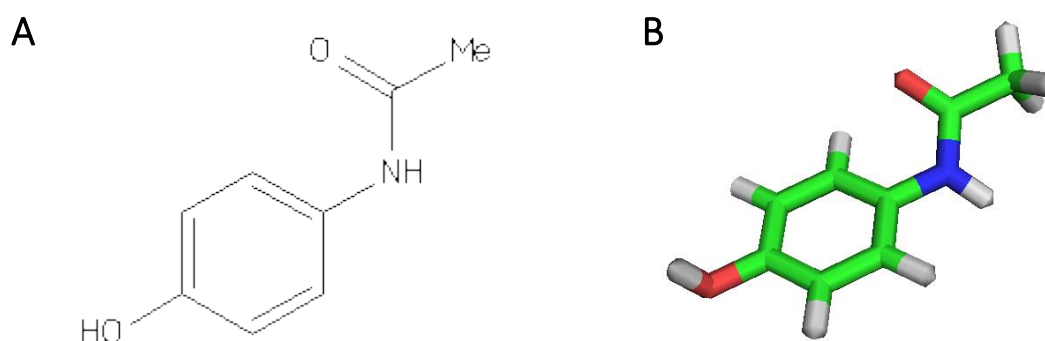
A

B

Figure 1.1.A shows the simple two-dimensional structure of paracetamol (copied from zinc.docking.org). **Figure 1.1.B** displays the stick representation of paracetamol, in PyMOL (ZINC18274777). Green: carbon, white: hydrogen, red: oxygen, blue: nitrogen.
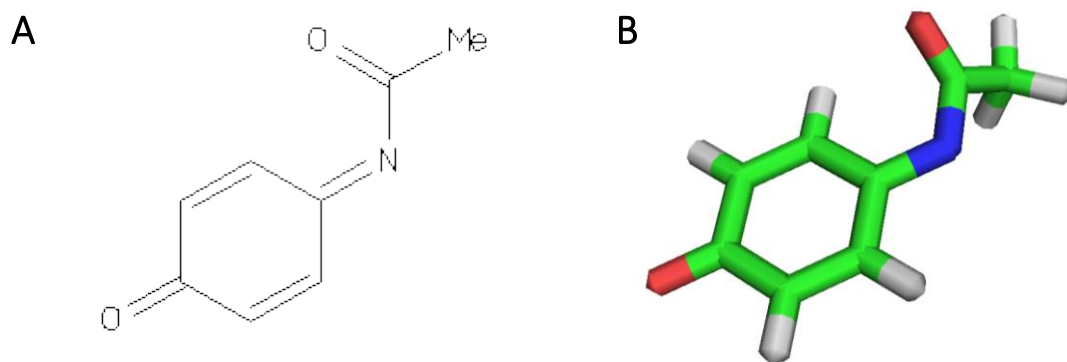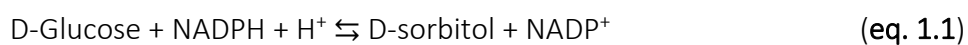
Figure 1.2.A shows the simple two-dimensional structure of NAPQT (copied from zinc.docking.org). **Figure 1.2.B** displays the stick representation of NAPQT, in PyMOL (ZINC02005673). Green: carbon, white: hydrogen, red: oxygen, blue: nitrogen.

1.2   D-sorbitol

D-sorbitol is a polyhydric alcohol that occurs naturally in different types of fruits and plants. It is also enzymatically produced from glucose in the glucose metabolism in the mammalian tissue, by aldose reductase:

$$D\text{-Glucose} + NADPH + H^+ \leftrightarrows D\text{-sorbitol} + NADP^+ \qquad \textbf{(eq. 1.1)}$$

D-sorbitol is often used as a sugar substitute in different types of food and beverages, as it is a better alternative in order to control diabetes and preserve dental health [3]. The structure of D-sorbitol is shown in figure 1.3 (A and B).
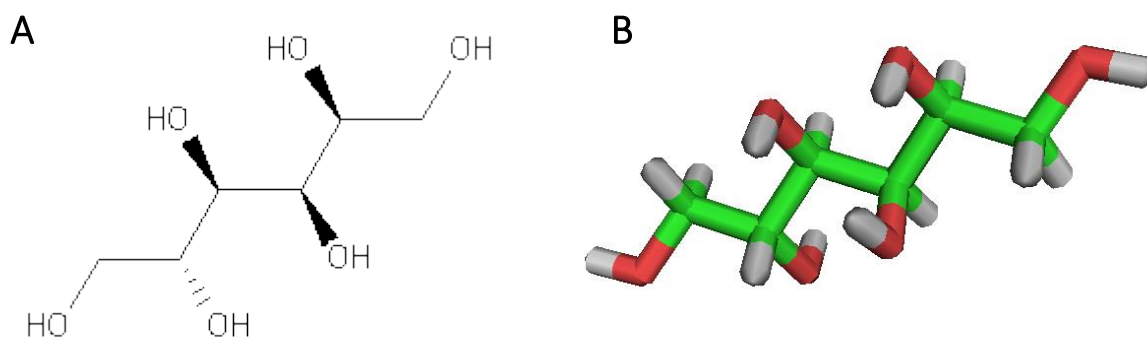

Figure 1.3.A shows the simple two-dimensional structure of one of the conformations of D-sorbitol (copied from zinc.docking.org). **Figure 1.3.B** displays the stick representation of D-sorbitol, in PyMOL (ZINC18279893). Green: carbon, white: hydrogen, red: oxygen.

1.3   Structure and mechanism of sorbitol dehydrogenase

Sorbitol dehydrogenase (SDH) is a NAD^+ dependent zinc tetrameric enzyme that catalyzes the following reaction [4]:

$$\text{D-Sorbitol} + NAD^+ \rightleftarrows \text{D-fructose} + NADH + H^+ \qquad \textbf{(eq. 1.2)}$$
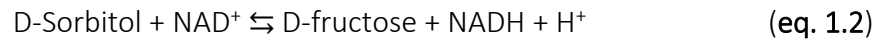
The focus in this thesis will be on the reaction where D-sorbitol is oxidized to D-fructose (left to right in eq. 1.2). The maximum rate for this reaction is at pH 9.9. [5].

 SDH is a tetramer of identical subunits, each binding a catalytic zinc atom and, when present, a coenzyme molecule; NAD^+/ NADH. [4]

The sequence of the 356 amino acids as well as the three-dimensional structure of the enzyme has been solved. The crystal structures of human SDH with NAD+, SDH with NADH and SDH with a fructose competitive inhibitor, has also been determined. The catalytic zinc is coordinated by His69, Cys44, Glu70, and a water molecule. [6].

The main folding of the enzyme-coenzyme complex of human SDH and NAD^+ is shown in figure 1.4 and 1.5, at different angles. Chain C of the complex of human SDH and NAD^+ is shown in figure 1.6, for a better look at the active site on the subunit, where the catalytic zinc atom is located. All the four subunits are identical, so the other chains would be similar.

SDH is one of the therapeutic targets for diabetic complications as it has been suggested that overproduction of NADH by SDH, and the redox changes this leads to, might play a part in dysfunction in sensitive tissues with the diabetic [6].

The K_M value of the reaction described by eq. 1.2 is estimated to be 1.8 mM at pH 9.9 [4].

Figure 1.4 shows the main folding of the enzyme-coenzyme complex of human SDH and NAD$^+$, in PyMOL (PDB ID: 1PL8). Chain A: green, chain B: orange, chain C: yellow, Chain D: red, NAD$^+$: blue, Zn: pink.
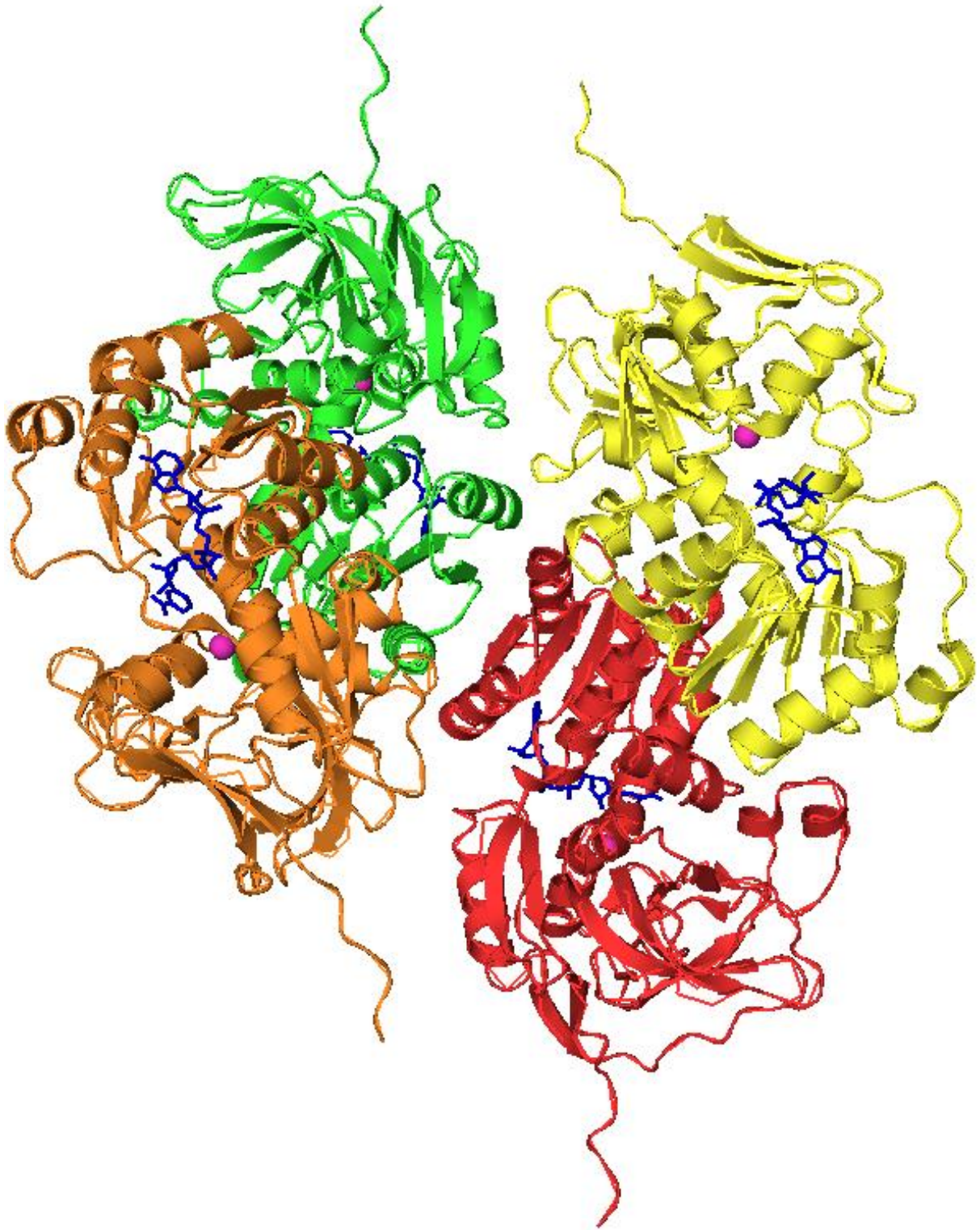
Figure 1.5 shows the main folding of the enzyme-coenzyme complex of human SDH and NAD⁺, in PyMOL (PDB ID: 1PL8). Chain A: green, chain B: orange, chain C: yellow, Chain D: red, NAD⁺: blue, Zn: pink.
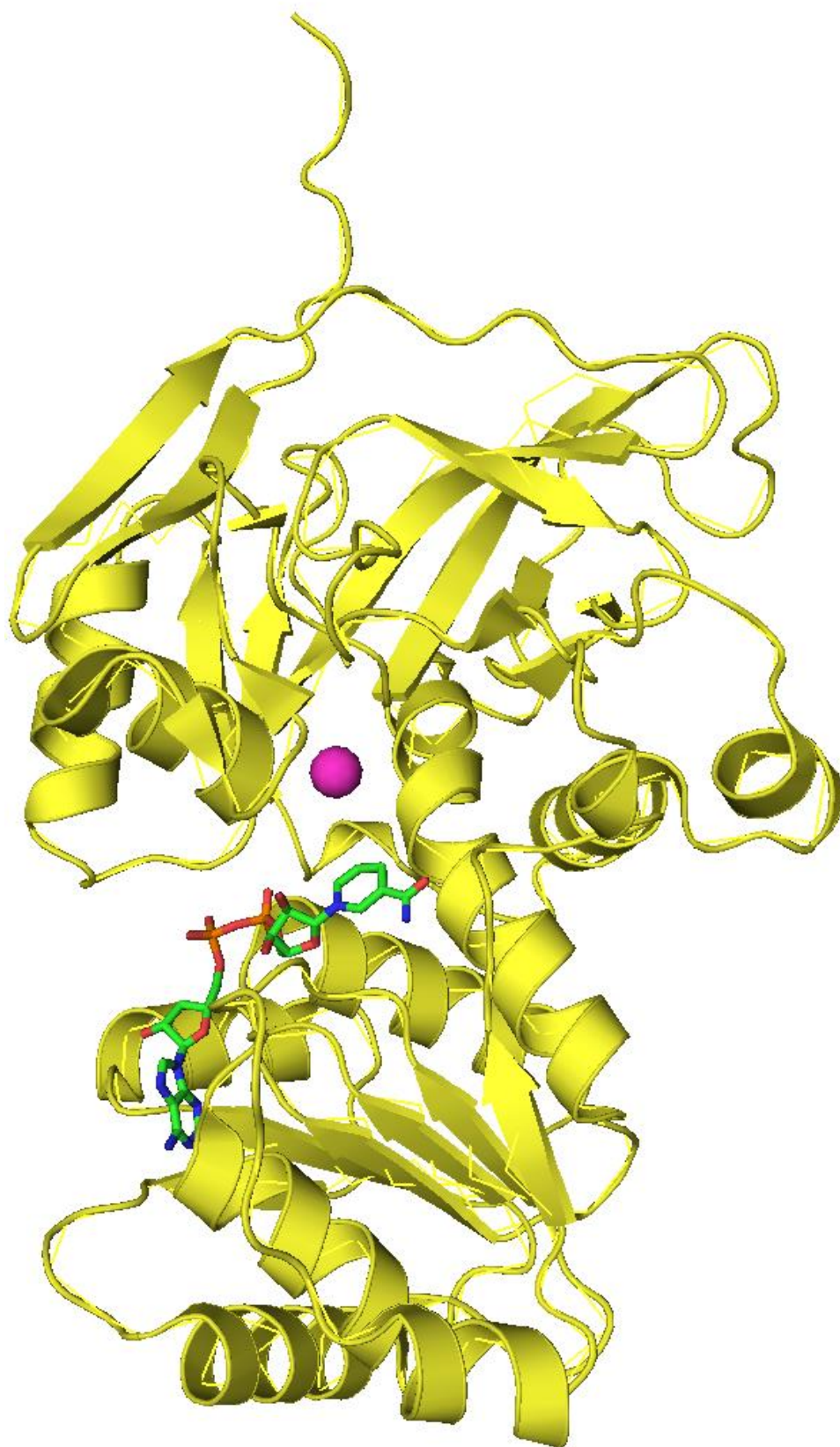
Figure 1.6 shows chain C of the enzyme-coenzyme complex of human SDH and NAD$^+$, in PyMOL (PDB ID: 1PL8). Chain C: yellow. NAD$^+$: green: carbon, blue: nitrogen, red: oxygen, orange: phosphor. Zn: pink.

2   Theory

2.1 Enzyme kinetics

Enzymes are proteins that catalyze all chemical processes in living organisms. The enzymes promote reactions without being consumed themselves. They are highly precise and efficient biomolecules, and life is said to be a result of enzyme catalyzed reactions. The enzymes do not influence the equilibrium of the reactions, they merely increase the reaction rate by lowering the reactions' energy barrier [7].

Many enzyme-catalyzed reactions follow a so called Michaelis-Menten kinetics. In all such cases, a specific substrate binds to an active site on the enzyme where the reaction occurs, and then there is formed an enzyme-substrate complex, ES. Product, P, is formed, and released from the enzyme. The enzyme is then again available to bind a new substrate. This can be expressed with the following equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P \qquad \text{(eq. 2.1)}$$
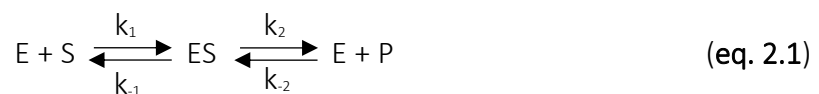
The Michaelis-Menten equation expresses the relationship between the rate of the reaction (v) and the substrate concentration [S]:

$$v = \frac{V_{max}[S]}{K_M + [S]} \qquad \text{(eq. 2.2)}$$

V_{max} is the maximum rate, and the K_M value indicates how great the affinity is between the enzyme and the substrate, and reflects the stability of the ES complex. The Michaelis-Menten equation assumes that there is a one-substrate reaction, which means that only one substrate can bind to the enzyme during the course of the reaction [7].

To find V_{max} and K_M the initial rates of a series of experiments, with constant enzyme concentration but with increasing substrate concentration, can be measured. That way, v as a function of [S] can be retrieved. To obtain V_{max} and K_M from this data, Lineweaver-Burk plot is most commonly used. Here, $\frac{1}{v}$ is plotted against $\frac{1}{[S]}$, giving a straight line (see figure 2.1 and eq. 2.3) [7].

When equation 2.2 is inversed, such as in Lineweaver-Burk, the new equation will be

$$\frac{1}{v} = \left(\frac{K_M}{V_{max}}\right)\left(\frac{1}{[S]}\right) + \frac{1}{V_{max}} \qquad \text{(eq. 2.3)}$$

This gives $\frac{1}{v} = \frac{1}{V_{max}}$ when $\frac{1}{[S]} = 0$ (see figure 2.1). To find K_M, the plot is extrapolated to the

point where $\frac{1}{v} = 0$. Then the following equation can be obtained:

$$\frac{1}{[S]_0} = \frac{-1}{K_M} \qquad \text{(eq. 2.4)}$$

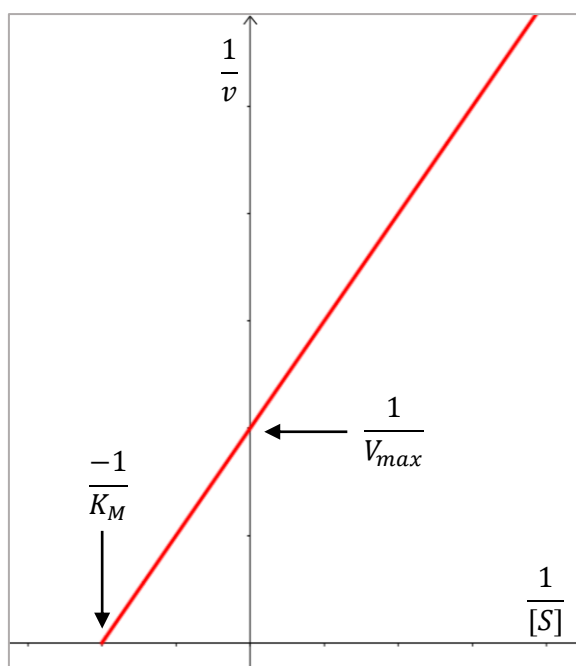In this equation $[S]_0$ is the value of [S] at $\frac{1}{v} = 0$ (see figure 2.1) [7].

Figure 2.1 shows a Lineweaver-Burk plot, where 1/v is graphed versus 1/[S]. It also shows how K_M and V_{max} is extrapolated from the data.

2.2 Factors that affect the enzyme activity

The enzyme activity can be affected by different factors like temperature, pH, ionic strength and enzyme inhibitors [8].

By adding heat, the reaction rate of the enzyme-catalyzed reaction will increase. Too much heat might lead to changes in the three-dimensional structure of the enzyme, or denaturation. Then the catalytic activity would decrease. Every enzyme has a temperature optimum, which is the temperature where the enzyme has an optimal activity. Temperatures above this will decrease the enzyme activity [8].

The enzymes contain acid and base groups that are affected by the change of pH. This affect can lead to the change of enzyme conformation, or the change of charge on the amino acids, which again influences the enzyme activity. The pH-optimum for the enzyme activity is the pH value that gives the highest reaction rate in an enzyme catalyzed reaction [8].

The ionic strength of the medium surrounding the enzymes has also an effect on the enzyme activity. Ions in the medium can neutralize the charges of the amino acids on the surface of the protein. This can have a great effect of the solubility of the proteins, by either decreasing or increasing it, depending on the characteristic of the enzyme surface. If the solubility decreases, the enzyme activity will decrease with it. If the solubility increases, so will the enzyme activity [8].

The enzyme activity can be inhibited by molecules that binds themselves to the enzyme. These molecules, the inhibitors, can be divided into two groups: irreversible and reversible. The irreversible inhibitors bind, often covalent, to a functional group on the enzyme that has a great importance to the activity [7].

There are several forms of reversible enzyme inhibition; competitive inhibition, uncompetitive, mixed and noncompetitive inhibition. Reversible inhibition means the involvement of an inhibitor where the effect on the enzyme can be reversed by removing the inhibitor. This is because the inhibitor binds noncovalent to the enzyme [7].

A competitive inhibitor resembles the substrate such that the enzyme will accept and bind it in its active site, but the inhibitor cannot be processed by the enzyme. This way the inhibitor will

compete against the substrate for the active sites, which means that the enzyme will not bind the substrate as well in the presence of the inhibitor. This will not have any affect in the V_{max} of the reaction, but will increase the K_M value (see figure 2.2) [7].

A noncompetitive inhibitor binds, not to the active site, but to a secondary site on the enzyme. This means that the substrate and the inhibitor can bind to the enzyme simultaneously, but the inhibitor slows the rate of product formation. V_{max} decreases because of this, while K_M will stay unaffected (see figure 2.3) [7].

Inhibitors that bind both the free enzyme E and the enzyme/substrate-complex ES, are called mixed inhibitor. A mixed inhibitor changes both the V_{max} and K_M values (see figure 2.5). Uncompetitive inhibitors bind only to the ES complex, and when it is bound, the substrate will not undergo any changes. Uncompetitive inhibitors also change both the V_{max} and K_M values (see figure 2.4) [13].

Figure 2.2 shows hos a competitive inhibitor alters the K_M value, while the V_{max} value stays unaffected.
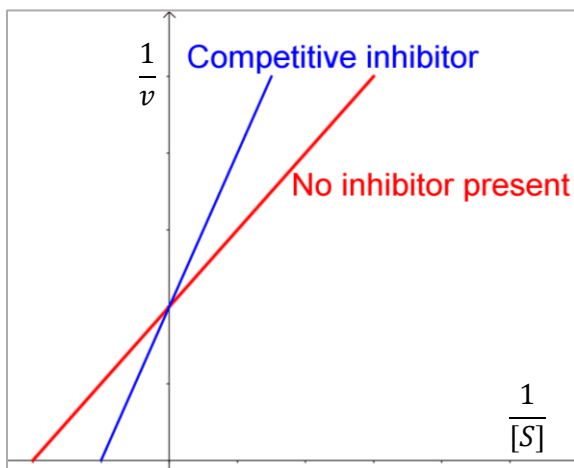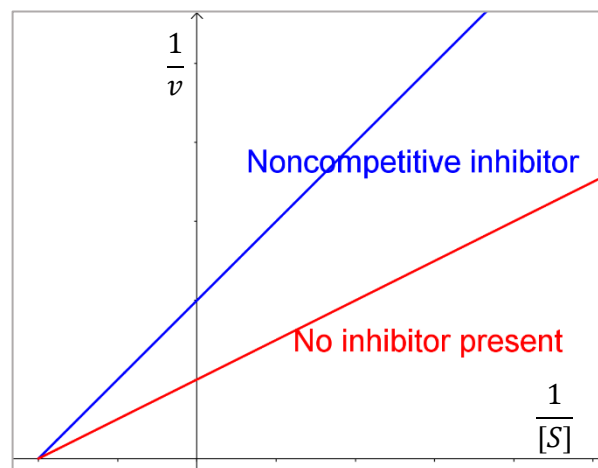

Figure 2.3 shows hos a noncompetitive inhibitor alters the V_{max} value, while the K_M value stays unaffected.
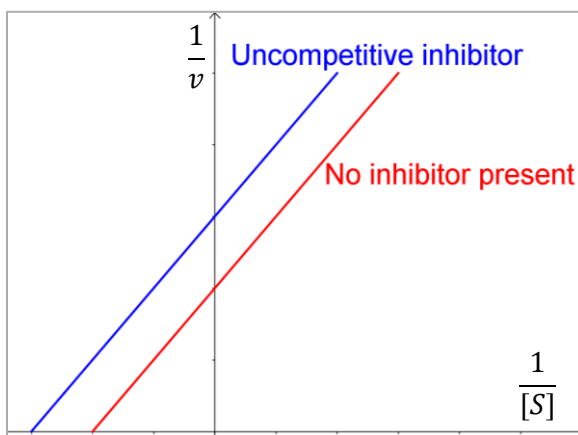

Figure 2.4 shows how a noncompetitive inhibitor affects the kinetics of an enzyme-catalyzed reaction.
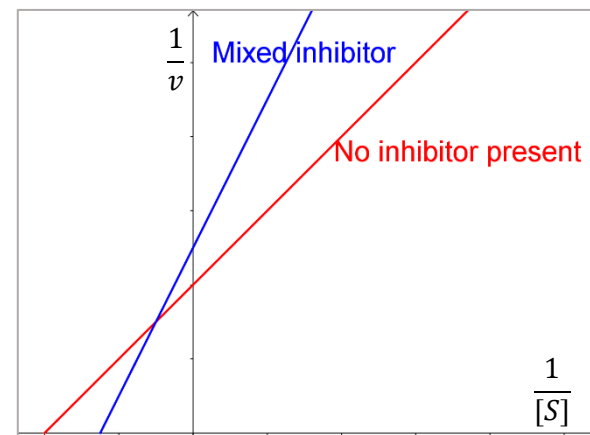

Figure 2.5 shows how a mixed inhibitor affects the kinetics of an enzyme-catalyzed reaction.

2.3 UV Spectroscopy

Spectrophotometric methods can be used to measure the rates of enzyme-catalyzed reactions. If these methods are to be used, one, and only one, of the participants in the reaction must absorb light in a specific area of the spectral region. In reactions where NADH is generated, the reaction rate can be measured by following the change in the absorbance by NADH, as NADH absorbs quite strongly at 340nm, while NAD$^+$ does not [7].

The change in absorbance by NADH can be measured by a method where light at 340nm is emitted through a cuvette containing the reaction solution, and the absorbance is measured over a period after the enzyme is added to the solution. As the concentration of NADH increases, the absorbance at 340nm will increase proportionally, per Beer-Lamberts law.

Beer-Lamberts law says that the absorbance (A) in a solution is proportional to the concentration (c) of the solution and to the length of the light's pathway (l) through the solution (eq. 2.5).

$$A = \varepsilon \cdot l \cdot c \qquad \text{(eq. 2.5)}$$

Here, ε is the molar absorption coefficient to the substance that absorbs at the given wave length, and l is the path length of the cuvette.

2.4 Computation

The Protein Data Bank (PDB; rcsb.org) is an archive that contains protein structures [11].

ZINC (zinc.docking.org) is a free database of compounds for virtual screening. The files from this library can be downloaded in the file format MOL2 [9]. This file format, along with the PDB file format, is compatible with both the molecular graphics system PyMOL [12], that is widely used to visualize molecules, and AutoDock Tools, both of which has been used in the work of this thesis.

AutoDock Vina is a software for executing molecular docking [10]. If given a receptor, a ligand, and a search area, Autodock Vina will be able to predict a possible receptor/ligand-complex and the binding affinity value of the complex. The receptor and ligand must be in the file format PDBQT, and is therefore converted to PDBQT by Autodock Tools. The reason why it must be a PDBQT file instead of PDB, is that this file contains hydrogens, and that is essential for the docking.

3 Materials and methods

1M NaOH	Sodium hydroxide	Merck
$NaH_2PO_4 \times 2H_2O$	Sodium phosphate monobasic dihydrate	Merck
$Na_2HPO_4 \times 2H_2O$	Disodium phosphate dibasic dihydrate	Merck
Glycine		Sigma
SDH	Sorbitol dehydrogenases from sheep liver	Sigma-Aldrich
NAD+	β-Nicotinamide adenine dinucleotide hydrate	Sigma-Aldrich
D-sorbitol		Sigma

To prevent contamination, all equipment was washed in concentrated nitric acid and then rinsed with milliQ water before use.

3.1 The phosphate buffer

The phosphate buffer pH 7.4, 0.1 M, was made by 3.12 grams of $NaH_2PO_4 \times 2H_2O$ and 14.24 grams of $Na_2HPO_4 \times 2H_2O$ mixed with milliQ water to the total volume of 1 liter.

3.2 The glycine buffer

The glycine buffer, 0.1M, was made by dissolving 7.3 grams of glycine in 800 deciliters of milliQ water, and then titrated with 1M NaOH until the desired pH value of 9.9 was reached. Then the volume was adjusted to a total of 1 liter.

3.3 Enzyme preparations

Cellulose membrane, 12 cm, was drained in milliQ water for a period of minimum two days.

The sorbitol dehydrogenase was bought as a lyophilizate, that contained approximately 8.3 mg solid, that consisted of both protein and the stabilizer, maltose. About 2 or 3 mg of this lyophilized powder was dissolved in 1 mL of glycine buffer, pH 9.9, and then quickly transferred to the cellulose membrane. A knot was made about 4 cm from the top, and the membrane was then attached to a 1 Liter Erlend Meyer flask, containing 1 liter of phosphate buffer, leaving the part of the membrane containing the enzyme completely drenched in the phosphate buffer. The enzyme was then dialyzed for three days, at 4ºC. The phosphate buffer was changed every day.

Then the enzyme was transferred to a tube of cellulose nitrate, and then kept on ice to prevent denaturation.

3.4 The enzyme kinetic experiment

To see whether paracetamol inhibits SDH or not, V_{max} and K_m was decided for the reaction

$$D\text{-sorbitol} + NAD^+ \rightarrow D\text{-fructose} + NADH + H^+ \qquad \text{(eq. 3.1)}$$
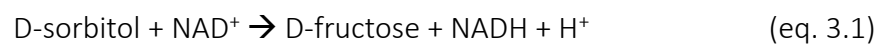
both with paracetamol present and without.

Four series of 10 reaction samples was made. Serie A had no paracetamol. Serie B, C and D contained respectively 0.05, 0.1 and 0.5mM paracetamol (see table 3.1). The 10 reaction samples in each series had increasing D-sorbitol concentration (see table 3.2). In addition to D-sorbitol and paracetamol, each reaction sample contained 50mM NAD⁺ and glycine buffer to a maximum volume of 990μL.

Table 3.1 shows the four series, and the paracetamol concentration in each.

Serie	A	B	C	D
[Paracetamol] (mM)	0	0.05	0.1	0.5

Table 3.2 shows the concentration of D-sorbitol in each of the ten reaction samples.

Reaction sample	1	2	3	4	5	6	7	8	9	10
[Sorbitol] (mM)	0.18	0.3	0.6	1.2	1.8	3.6	5.7	9	13.5	18

Each reaction sample was placed in a quartz cuvette, with the path length of 1 cm, and then inserted into the spectrophotometer (HITACHI U 2000). After the spectrophotometer was auto zeroed, 10μL of enzyme was mixed into the sample, and the spectrophotometer measured the change of absorbance by NADH at 340nm every ten seconds over 2 minutes. This gave the initial velocity of the reaction in the cuvette, at 23°C. The settings of the HITACHI U 2000 are shown in table 3.3.

Table 3.3 show the settings that was used on the HITACHI U 2000 Spectrophotometer.

DATA MODE	RATE ASSAY
WL (nm)	340.0
UP SCALE	0.500
LO SCALE	0.000
INIT DELAY (sec)	0
SCAN TIME (sec)	120
CALC DELAY	0
CALC TIME	120
RATE FACTOR	1.000
DISPLAY FORMAT	SEQUENTIAL
BASELINE	USER
RESPONSE	MEDIUM
LAMP CHANGE WL (nm)	340.0
VIS LAMP	ON
UV LAMP	ON
GRAPH PRINT	ON
TECT PRINT	ON
LIST INTERVAL (sec)	10

To better see the influence paracetamol had on the reaction, a series E was made, consisting of six reaction samples with constant D-sorbitol concentration at 5mM, but increasing paracetamol concentration (see table 3.4). In addition to D-sorbitol and paracetamol, each reaction sample contained 50mM NAD^+ and glycine buffer to a maximum volume of 990μL. These samples were measured spectrophotometrically at 340nm the same way as the samples from series A-D.

Table 3.4 shows the concentration of D-sorbitol in each of the ten reaction samples in series E.

Reaction sample	1	2	3	4	5	6
[Sorbitol] (mM)	0.5	0.8	1.25	2	3.15	5

3.5 Prediction of binding

The program AutoDock Vina was used to predict the binding of paracetamol to SDH. The information the program used to predict the binding is given in table 3.5. The grid box size and location was constructed by adapting it into the biggest gap in chain C on the SDH, in AutoDock Tools. Chain C was chosen randomly from the four chains and, since the chains are identical, it is assumed that the results given from chain C would be similar to the results from any of the other chains.

Table 3.5 shows the information given to the AutoDock Vina program.

Molecules	Receptor	SDH
	Ligand	Paracetamol
Grid box	Center_x	46.304
	Center_y	16.706
	Center_z	10.806
	Size_x	20
	Size_y	20
	Size_z	25

4   Results

4.1   The enzyme kinetic experiment

Four series of 10 reaction samples were prepared. The four series A, B, C and D had respectively 0, 0.05, 0.1 and 0.5mM paracetamol. The 10 samples in each series had increasing D-sorbitol concentration. All samples were measured spectrophotometrically at 340nm every ten seconds over 2 minutes (see table 3.3).

The difference in A_{340} the first 10 seconds ($dA_{340}/10s$) was multiplied by 6, to find dA_{340}/min. This value is used as an approach to the initial reaction rate, v, in each sample. The results are found in table 4.1.

The $dA_{340}/10s$ values are given in Appendix I, in table I.A.

Table 4.1 shows the calculated initial reaction rate (v) for each of the 10 reaction samples in the four series.

Reaction sample	[Sorbitol] (mM)	Serie A v (dA_{340}/min)	Serie B v (dA_{340}/min)	Serie C v (dA_{340}/min)	Serie D v (dA_{340}/min)
1	0.18	0.03	0.018	0.018	0.006
2	0.3	0.042	0.006	0.03	0.018
3	0.6	0.072	0.06	0.066	0.036
4	1.2	0.12	0.072	0.036	0.06
5	1.8	0.15	0.09	0.108	0.036
6	3.6	0.186	0.072	0.108	0.06
7	5.7	0.252	0.228	0.084	0.054
8	9	0.162	0.252	0.198	0.294
9	13.5	0.33	0.126	0.21	0.3
10	18	0.336	0.072	0.33	0.264

Lineweaver-Burk plot was used to analyze the results, where $\frac{1}{v}$ was plotted against $\frac{1}{[Sorbitol]}$. The values of $\frac{1}{v}$ and $\frac{1}{[Sorbitol]}$ are displayed in table 4.2. The double inverse plot of each of the series is shown in figure 4.1. Some of the values in table 4.2 are not included in the graphic representation of the results in figure 4.1, as these values are outliers.

Table 4.2 shows the inverse values of both the D-sorbitol concentrations and initial velocity for all four series.

Reaction sample	$\dfrac{1}{[Sorbitol]}$ (mM^{-1})	Serie A $\dfrac{1}{v}$ $(dA_{340}/min)^{-1}$	Serie B $\dfrac{1}{v}$ $(dA_{340}/min)^{-1}$	Serie C $\dfrac{1}{v}$ $(dA_{340}/min)^{-1}$	Serie D $\dfrac{1}{v}$ $(dA_{340}/min)^{-1}$
1	5.56	33.33	55.56	55.55	166.67 *
2	3.33	23.81	166.67 *	33.33	55.55
3	1.67	13.89	16.67	15.15	27.78
4	0.83	8.33	13.89	27.78 *	16.67
5	0.56	6.67	11.11	9.26	27.78 *
6	0.28	5.38	13.89 *	9.26 *	16.67 *
7	0.18	3.97	4.39	11.9 *	18.52 *
8	0.11	6.17 *	3.97	5.05	3.4
9	0.07	3.03	7.94 *	4.76	3.33
10	0.05	2.98	13.89 *	3.03	3.79

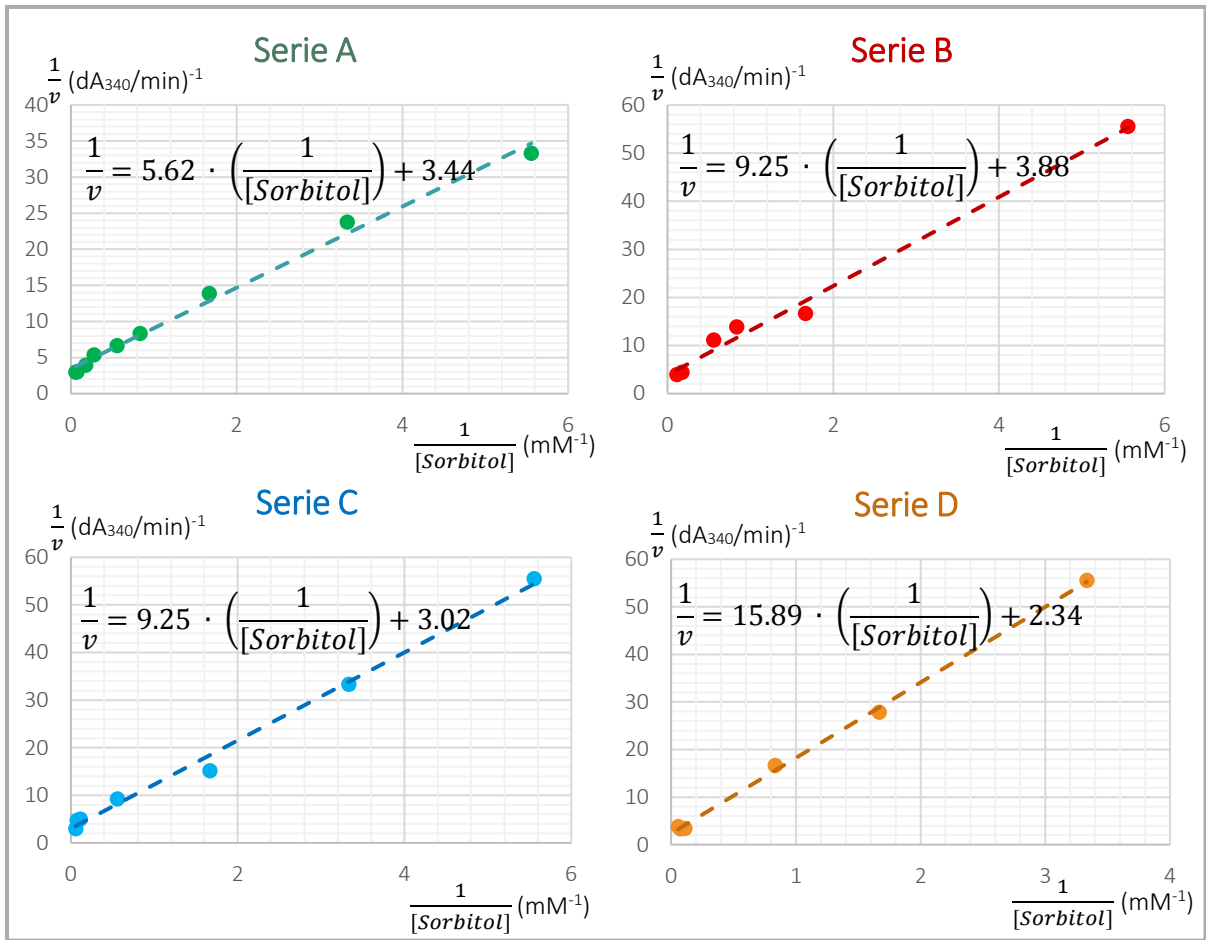* values that are excluded from the graphic representation in figure 4.1.

Figure 4.1 shows the double inverse plot for each of the four series, A-D, separately. The equation for each of the lines are also shown in the graph area.
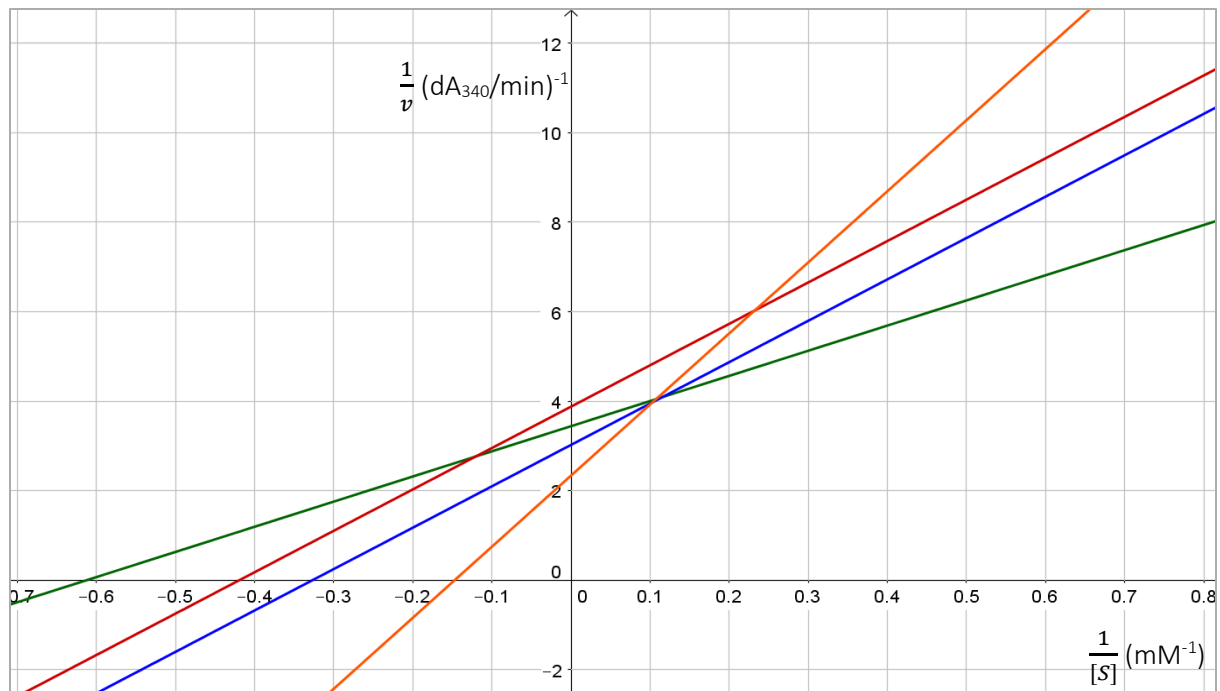

Figure 4.2 shows the graphic representation of the fours series, A-D, in the same coordinate system. Serie A: green. Serie B: Red. Serie C: blue. Serie D: orange. The equations of the lines are shown in figure 4.1.
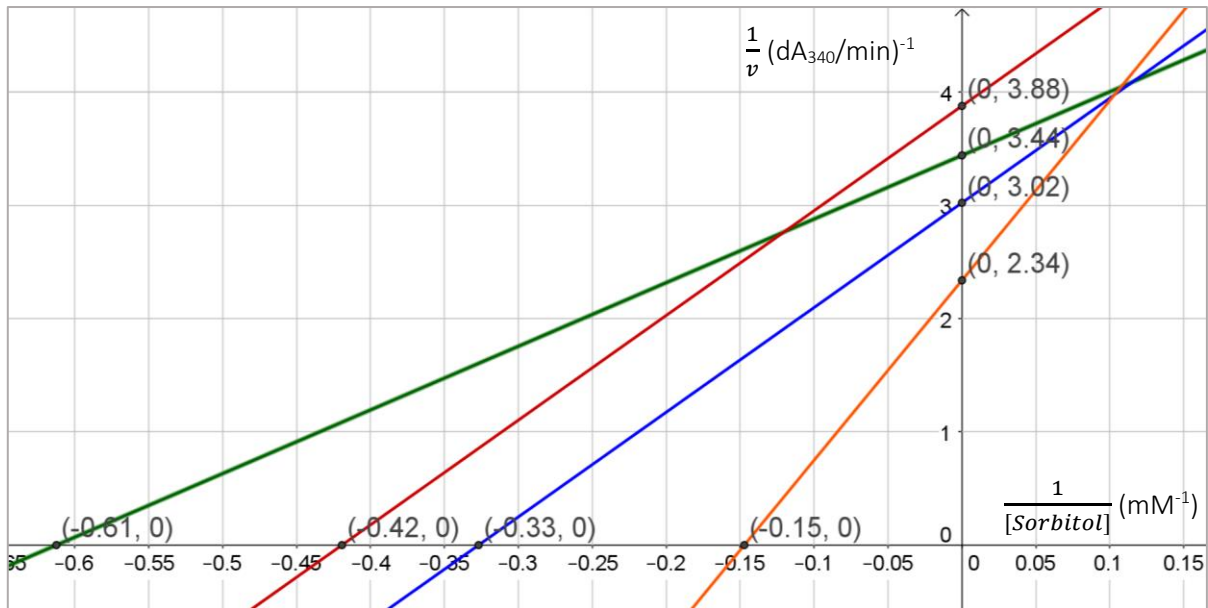
Figure 4.3 shows the intersections between the linear functions and the axis.

The intersections between the linear functions and the axis in figure 4.3 gives the values of $\frac{1}{V_{max}}$ and $\frac{-1}{K_M}$ for each of the four series, according to figure 2.1. The values of $\frac{1}{V_{max}}$ and $\frac{-1}{K_M}$ for each of the four series, and the calculated values for V_{max} and K_M, are listed in table 4.3.

The graphic representation of the four series shown in figure 4.2 and 4.3 show that both the lines of series C and D crosses the line of series A at approximately the same point. The line representing the series with the lowest concentration of paracetamol, series B, differs from the other two.

The results show that both V_{max} and K_M for the reaction (eq. 1.2) changes in the presence of paracetamol. The higher the concentration of paracetamol is, the higher the K_M value gets.

The V_{max} value of series B is lower than for series A, while both series C and D has a higher V_{max} value.

Table 4.3 shows the $\frac{1}{V_{max}}$ and $\frac{-1}{K_M}$ values obtained from the graphic representation of the four series (see figure 4.3), and the calculated values of V_{max} and K_M.

Serie	[Paracetamol]	$\frac{1}{V_{max}}$	V_{max}	$\frac{-1}{K_M}$	K_M
	(mM)	(dA340/min)$^{-1}$	(dA340/min)	(mM^{-1})	(mM)
A	0	3.44	0.291	-0.61	1.639
B	0.05	3.88	0.258	-0.42	2.381
C	0.1	3.02	0.331	-0.33	3.03
D	0.5	2.34	0.427	-0.15	6.667

A series E of six reaction samples with constant D-sorbitol concentration at 5mM, but increasing paracetamol concentration, were prepared. All six samples were measured spectrophotometrically at 340nm every ten seconds over 2 minutes.

The difference in A_{340} the first 10 seconds ($dA_{340}/10s$) was multiplied by 6, to find dA_{340}/min. This value is used as an approach to the initial reaction rate, v, in each sample. The results are found in table 4.4.

Figure 4.4 shows the plot of the initial rate against the concentration of paracetamol. One of the values in table 4.4 is not included in the plot in figure 4.4, as this value is an outlier.

Table 4.4 shows the paracetamol concentration of each reaction sample in series E, and the calculated initial velocity, v.

Reaction sample	[Paracetamol] (mM)	v (dA_{340}/min)
1	0.5	0.138
2	0.8	0.104
3	1.25	0.078
4	2	0.066
5	3.15	0.234 *
6	5	0.024

* values that are excluded from the graphic representation in figure 4.4.
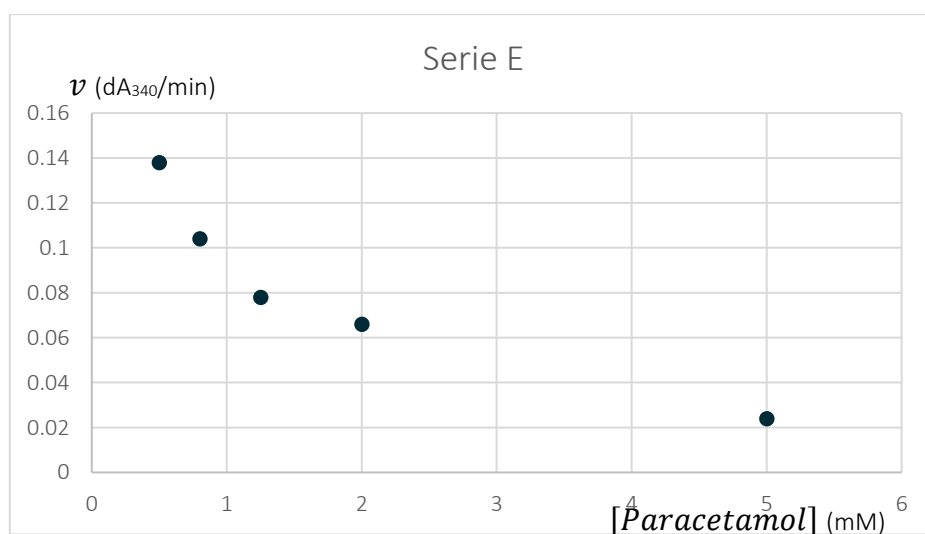

Figure 4.4 shows the plotted v against [paracetamol] of series E.

4.2   Docking results

AutoDock Vina was used to predict a possible receptor/ligand-complex and the binding affinity of this. Figure 4.5 shows a screenshot of the output written from AutoDock Vina, and it shows the affinity for 9 different SDH/paracetamol-complexes, and that they are all negative. Negative affinity gives binding. The SDH/paracetamol–complex with the most negative affinity, and therefore the strongest binding, is shown in figure 4.6. A close-up to the binding site is shown in figure 4.7.

```
mode |   affinity | dist from best mode
     | (kcal/mol) | rmsd l.b.| rmsd u.b.
-----+------------+----------+----------
   1*        -5.1       0.000       0.000
   2         -4.9       3.270       5.377
   3         -4.9       2.169       4.784
   4         -4.7       2.048       2.589
   5         -4.7       3.809       5.176
   6         -4.5       2.756       3.212
   7         -4.3       4.147       6.429
   8         -4.3       7.301       8.295
   9         -4.2       2.529       4.958
```

Figure 4.5 Screenshot of the output written from AutoDock Vina.
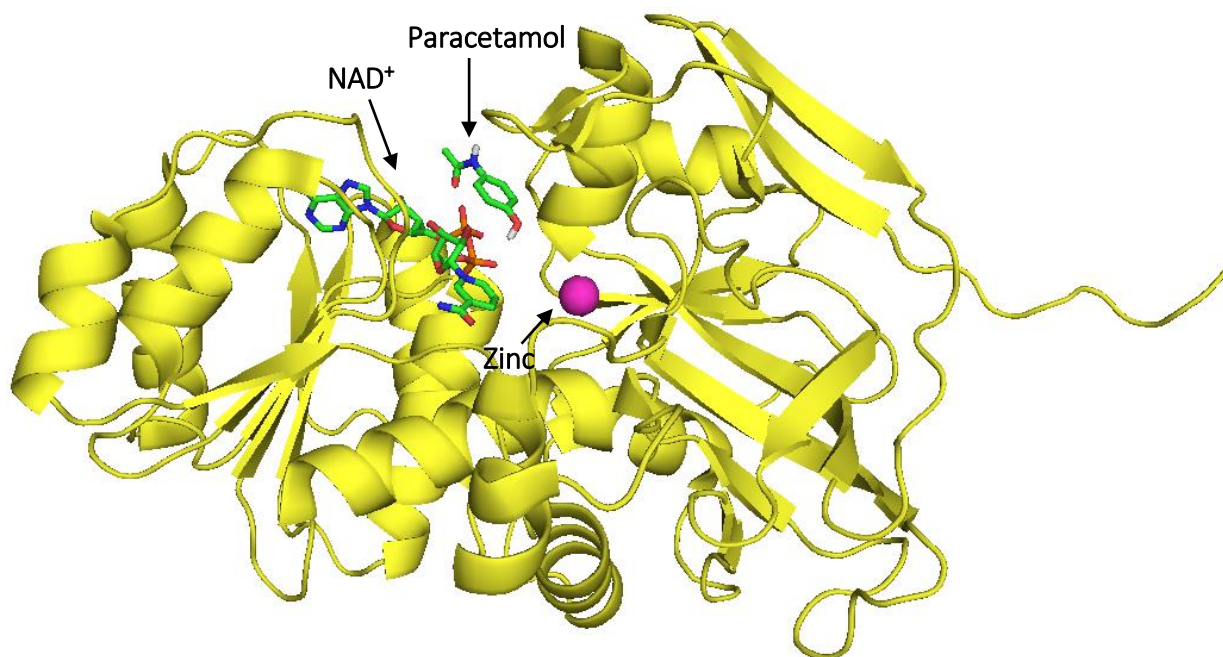*visualized in figure 4.6.

Figure 4.6 shows paracetamol docked in the active site of the C chain of the enzyme-coenzyme complex of human SDH and NAD$^+$, in PyMOL (PDB ID: 1PL8). Chain C: yellow. Paracetamol and NAD$^+$: green: carbon, blue: nitrogen, red: oxygen, orange: phosphor. Zn: pink.
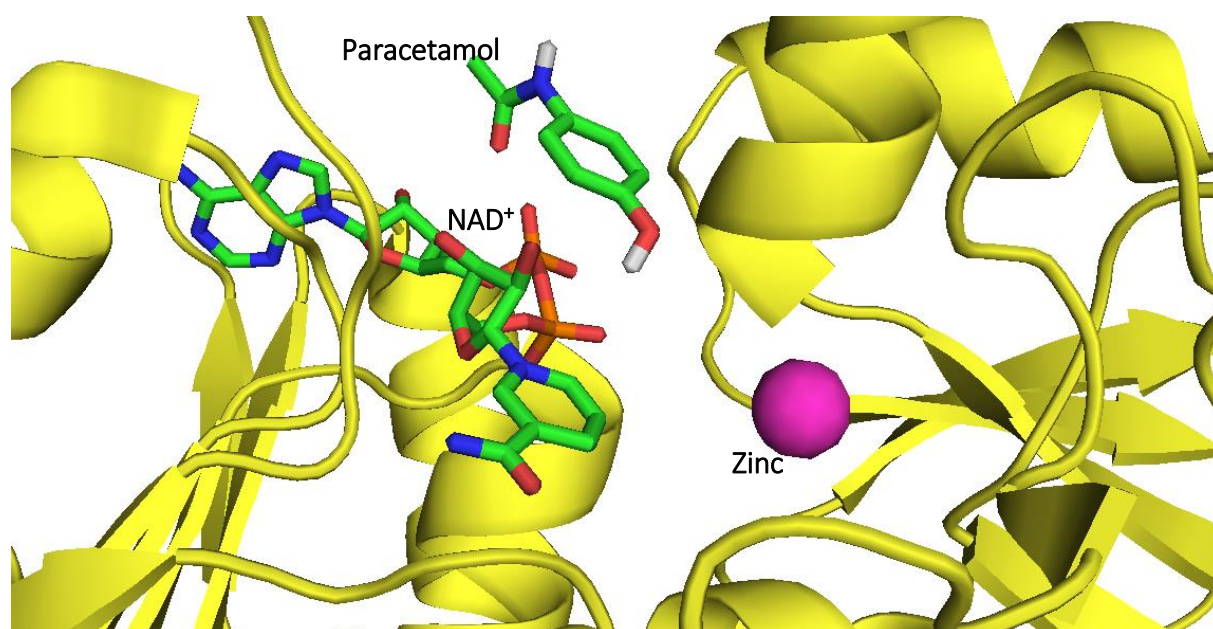

Figure 4.7 shows a close-up of paracetamol docked in the active site of the chain C of the enzyme-coenzyme complex of human SDH and NAD$^+$, in PyMOL (PDB ID: 1PL8). Chain B: yellow. Paracetamol and NAD$^+$: green: carbon, blue: nitrogen, red: oxygen, orange: phosphor. Zinc: pink.

5   Discussion

Sorbitol dehydrogenase catalyzes both the oxidation of D-sorbitol to D-fructose, and the reduction of D-fructose to D-sorbitol. The reaction where D-fructose is formed has the reaction rate optimum at pH 9.9, while the other reaction is favored at a lower pH (<7.4) [4]. Therefore, it is possible to assume that when D-sorbitol, NAD^+ and SDH is added to a medium of pH 9.9, the reaction (eq. 1.2) will mainly go to the right.

Since the reaction is pH dependent, it is important that the acidity does not change during the experiment, if the results are to be right. Therefor it is important that the buffer used can counteract moderate changes in pH. Glycine buffer is used, as this is a gentle medium that will have little effect on the characteristics of the enzyme. As high buffer concentration and ionic strength can inhibit the enzyme activity, the ionic strength of the buffers was kept low at 0,1M. This concentration has proven to be the most favorable, according to those who has worked with SDH earlier (ref. Lindstad).

Michaelis-Menten kinetics requires a one-substrate reaction, where only one specific substrate binds to the active site. In the case of SDH, both D-sorbitol and NAD^+/ NADH acts as substrates. This means that it is in fact a two-substrate reaction, and therefore Michaelis-Menten cannot be used initially. If, however, the solution is saturated with NAD^+, the reaction will work approximately as a one-substrate reaction, a so-called pseudo one-substrate reaction, and that makes it possible to use Michaelis-Menten.

The spectrophotometer used in the experiments in this thesis, is manual. It means that the chamber, where the cuvette is placed, must be opened to insert the enzyme. As soon as the enzyme is added to the reaction sample, the chamber must be closed, and then the instrument will start measuring the absorbance when the "start"-button is pushed. This is a weak link in an experimental method where the initial reaction rate is to be measured. If the reaction goes very fast the first five seconds after the enzyme is added, and then slows down, this might be lost by the time it takes to close the chamber and push the "start"-button. Also, it requires a great deal of practice and routine to be able to use the same amount of time from the enzyme is added, to the "start"-button is pushed, from sample to sample, so that each sample is equally measured. This can explain eventual "outliers" in a series of samples measured.

When the experiments were executed, it was unsure how the reaction would develop over time. Therefore, the spectrophotometer was set to measure the absorbance every ten seconds, over a period of 2 minutes. The absorbance changed quickly the first ten seconds, and then the reaction seemed to slow down a little. Therefore, only the measurements of the first ten seconds was used in the calculation of the initial rate of the reaction.

The results from the laboratory indicates that paracetamol could be an inhibitor of sorbitol dehydrogenase. Both from the graphic representations of the four series shown in figure 4.2 and 4.3, and the values listed in table 4.3, shows that both V_{max} and K_M for the reaction where D-sorbitol is oxidized to D-fructose by sorbitol dehydrogenase, changes in the presence of paracetamol. The higher the concentration of paracetamol is, the higher the K_M value gets. When the K_M value gets higher, the sorbitol dehydrogenase's affinity for the substrate D-sorbitol decreases. This means that paracetamol is lowering the affinity of the SDH/D-sorbitol-complex, which is typical for some kinds of inhibitors, especially those who bind to the active site.

It is normal when an enzyme is inhibited, that the V_{max} of the reaction it catalyzes is lowered, depending of course on the way the inhibitor works. It is, however, extremely uncommon that the V_{max} increases in the presence of an inhibitor. The results given in figure 4.2 and 4.3 and table 4.3 show that both the presence of paracetamol in series C and D, with the respective paracetamol concentrations 0.1 and 0.5mM, has the effect of increasing the V_{max} of the reaction. The meaning of this is not known. It cannot be excluded however, that this result is due to errors that could be made in the laboratory, as for example wrong concentrations of the stock solution of D-sorbitol.

The results given for series B differs from those given for series C and D. The results show that Vmax of the reaction is lowered when the concentration of paracetamol is 0.5mM, such as in series B. This is the series with the lowest concentration of paracetamol, and that could explain why it differs from the others. It might have a paracetamol concentration that is too low to affect the reaction like the other two.

The docking results supports the theory of paracetamol inhibiting SDH. The SDH/paracetamol-complex predicted to have the strongest binding was predicted to have an affinity value of -5.1 kcal/mol. In the results shown in figure 4.6 and 4.7, paracetamol goes toward the zinc atom

along with NAD^+. If paracetamol had functioned as an uncompetitive inhibitor or a noncompetitive inhibitor, this would not be the case. In those cases, the paracetamol would bind somewhere else on the enzyme, and not in the active site.

Paracetamol is expected to dock the same way as in chain C in all the chains of sorbitol dehydrogenase, as the four chains are identical, and should bind equally to the ligand.

So, both the kinetic data collected in the laboratory and the results given from docking in AutoDock Vina, indicates that paracetamol could be an inhibitor of D-sorbitol. It is uncertain what kind of inhibition this would be, as the results are a little difficult to interpret. It could be mixed inhibition, as it is not a clear competitive inhibition.

6 Conclusion

Both the kinetic and the docking data indicates that paracetamol binds to the active site, along with NAD^+/NADH, and acts as an inhibitor of SDH. It is uncertain what kind of inhibition this is, but the results might indicate a form of mixed inhibition.

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APPENDIX I

Table I.A shows the values of dA$_{340}$/10s for each of the 10 reaction samples in the four series.

Reaction sample	[Sorbitol] (mM)	Serie A dA$_{340}$/10s	Serie B dA$_{340}$/10s	Serie C dA$_{340}$/10s	Serie D dA$_{340}$/10s
1	0,18	0,005	0,003	0,003	0,001
2	0,3	0,007	0,001	0,005	0,003
3	0,6	0,012	0,01	0,011	0,006
4	1,2	0,02	0,012	0,006	0,01
5	1,8	0,025	0,015	0,018	0,006
6	3,6	0,031	0,012	0,018	0,01
7	5,7	0,042	0,038	0,014	0,009
8	9	0,027	0,042	0,033	0,049
9	13,5	0,055	0,021	0,035	0,05
10	18	0,056	0,012	0,055	0,044