Purification and Characterization of Bovine Liver Uridine Phosphorylase

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ABSTRACT

Uridine phosphorylase (UPP) is an enzyme that catalyzes the reversible phosphorylytic cleavage of uridine to uracil and ribose-1-phosphate.[1] A strong interest has been expressed in the enzyme in recent years because of the recognition of its many medicinal applications. Naturally occurring uridine has been shown to exhibit a cytoprotective effect against the toxicity associated with chemotherapy treatments for both oncological and infectious diseases.[2] Inhibition of UPP drastically slows the breakdown of uridine and therefore raises plasma uridine levels in mammals.

Determining a purification scheme is the first step to acquiring a pure enzyme that can be studied in the pursuit of the development of stronger and more specific UPP inhibitors. The purification scheme outlined in this thesis consisted of the following steps: ammonium sulfate precipitation, Mono Q ion exchange chromatography, Sephacryl S100 size exclusion chromatography, and hydroxyapatite chromatography. UPP was purified 64.24-fold with 4.01% final yield. The initial extract showed a specific activity of 0.41 nmoles/min/mg, which increased to 26.51 nmoles/min/mg after the final purification step. Additionally, the purified enzyme was found to exhibit cleaving activity for uridine, thymidine and inosine in the ratio of 5:1:0.

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CHAPTER I

INTRODUCTION

Uridine phosphorylase (UPP) is an enzyme that catalyzes the reversible phosphorylytic cleavage of uridine to uracil and α-ribose-1-phosphate (Figure 1).[1]

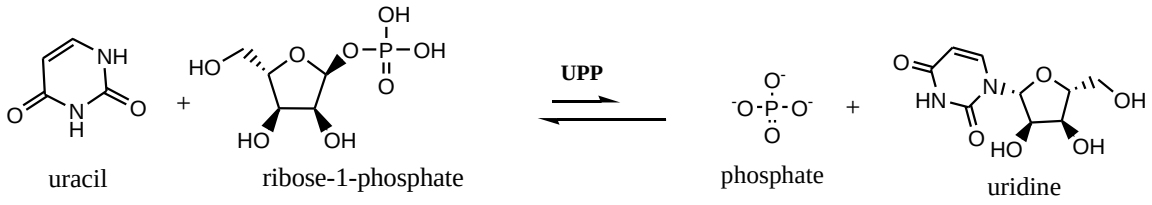


Figure 1. Reaction catalyzed by uridine phosphorylase in the nucleoside salvage pathway

The enzyme exists in all prokaryotes and eukaryotes and is essential in the nucleoside salvage pathway. It is part of a larger class of nucleoside phosphorylases including the purine nucleoside phosphorylases (inosine and adenosine phosphorylase) and the pyrimidine nucleoside phosphorylases (thymidine, cytidine, and uridine phosphorylase). Nucleoside phosphorylases are divided into two super families: NP-I and NP-II.[1] All known UPPs belong to the NP-I family, and although microorganisms exhibit a hexameric structure, this is reduced to a tetramer or dimer structure favored by evolution in higher-level organisms.[1] UPP was initially purified and studied in the prokaryotic organism *E.coli*, and has since sparked the interest of scientists around the world because of the discovery of its many medicinal applications. Naturally occurring uridine has been shown to exhibit a cytoprotective effect against the toxicity associated

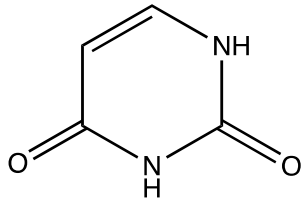
with chemotherapy treatments for both oncological and infectious diseases. Because UPP varies structurally among organisms, these differences could be exploited in the design of inhibitors selectively geared to impair UPP activity in microorganisms. This renders UPP an attractive target for the treatment of bacterial infections in higher-level animals, such as humans.[2] This introduction will explore some of the most prevalent areas of medicine that can be greatly improved by the acquired knowledge of UPP and explain why the development of improved, selective UPP inhibitors are necessary for the advancement of current oncological, antiviral, and antimicrobial medical therapies. In addition, the existing structural, functional, and biomedical information on the enzyme, from the primitive hexameric form initially isolated from *E.coli*, to the highly evolved dimer found in humans will be introduced.

1.1 UPP inhibitors used as modulators for nucleoside analog therapy

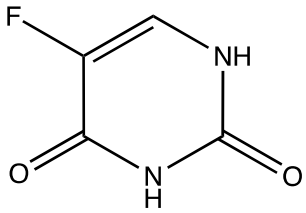
1.1.1 UPP Inhibitors in chemotherapy (uracil analogs)

Recently, there has been a focus on developing UPP inhibitors as a way to improve cancer treatment. According to the American Cancer Society, the estimated cancer prevalence in the United States as of January 1, 2010 is 13,028,000 people.[3] Globally, 7.6 million people die each year from cancer, which is more than the amount of people who die from tuberculosis, AIDS, and malaria combined. In addition, if attempts to increase effectiveness of available cancer treatments are unsuccessful, the amount of deaths caused by the disease annually around the world is projected to increase by 80% by 2030.[3] For the past several decades, the uracil analog 5-flourouracil (5-FU) has been administered to cancer patients as part of a chemotherapy regimen. 5-FU is an analog of natural uridine and primarily works to halt DNA and RNA synthesis by posing as uridine,

and therefore causing the affected tumor cells to undergo apoptosis.[4-6] Although structurally very similar, 5-FU will not carry out the necessary functions of uridine due to the fluorine moiety (Figure 2).



uracil



5-fluorouracil

Figure 2. Structures of 5-fluorouracil and uracil

In DNA, the uridine analog interrupts synthesis by inhibiting the enzyme thymidylate synthase (TS), which converts deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP). This blocks the formation of thymidine, an important nucleoside necessary for DNA replication.[7] These pathways are illustrated in Figure 3, beginning with capecitabine, which is an orally administered prodrug of 5-FU.

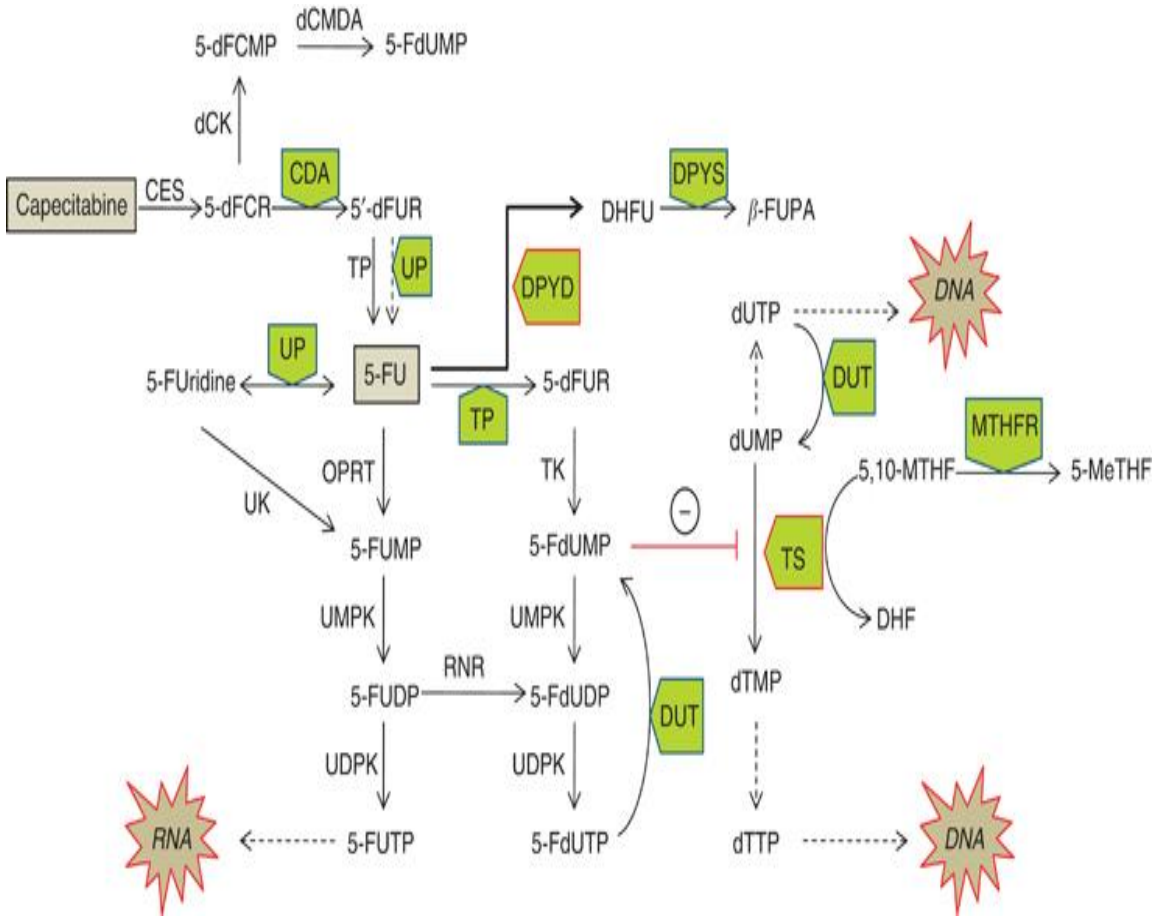


Figure 3. Metabolism of capecitabine and 5-FU Enzymes: Carboxyl esterase (CES), deoxycytidine kinase (dCK), deoxycytidine monophosphate deaminase (dCMDA), cytidine deaminase (CDA), thymidine phosphorylase (TP), uridine phosphorylase (UP), dihydropyrimidine dehydrogenase (DPYD), dihydropyrimidinase (DPYS), orotate phosphoribosyltransferase (OPRT), uridine kinase (UK), uridine monophosphate kinase (UMPK), uridine diphosphate kinase (UDPK), ribonucleotide reductase (RNR), thymidine kinase (TK), thymidine synthase (TS), deoxyuridine triphosphatase (DUT), methylene tetrahydrofolate reductase (MTHFR). Metabolites: deoxyfluorocytidine riboside (5′-dFCR), deoxyfluorocytidine monophosphate (5′-dFCMP), deoxyfluorouridine monophosphate (5-FdUMP), deoxyfluorouracil (5′-dFUR), fluorouracil (5-FU), fluorouridine (5-FUridine), fluorouracil monophosphate (5-FUMP), fluorouracil di, tri-phosphate (5-FUDP, 5-FUTP), deoxyfluorouracil di, tri-phosphate (5-FdUDP, 5-FdUTP), deoxyuridine mono, tri –phosphate (dUMP, dUTP), deoxycytidine mono, tri-phosphate (dTMP, dTTP), 5,10-methylenetetrahydrofolate (5,10-MTHF), 5-methyltetrahydrofolate (5-MeTHF), dihydrofolate (DHF), dihydrofluorouracil (DHFU), beta-fluoroureido propionic acid (β-FUPA). Reprinted with permission from Loganayagam A.; Hernandez M.A.; Corrigan A.; Fairbanks L.; Lewis C.M.; *et al.* Pharmacogenetic variants in the dpyd, tyms, cda and mthfr genes are clinically significant predictors of fluoropyrimidine toxicity. *Br. J. Cancer* **2013**, *108*, 2505-2515.

Although to an extent, these effects are widespread throughout the body and do not discriminate between cancerous and normal cells, the logic behind the treatment is that some degree of specificity towards cancerous cells can be achieved due to the inherently less stable nature of the rapidly dividing cancer cells. Because 5-FU is very similar in structure to uracil, the rapidly dividing cells have shown to be more likely to accept the analog than the more discriminating normal tissue cells. Also contributing to this phenomenon of selectivity is the existence of higher levels of UPP in tumor cells. UPP is involved in the pentose phosphate cycle, which plays an essential role in cell division and growth.[2] The more divisions a cell undergoes, the more it relies on the pentose phosphate cycle, and therefore the more strongly it needs UPP.[2] Hence, UPP activity is much higher in tumor tissue than in the surrounding tissue, as demonstrated in Figure 4.

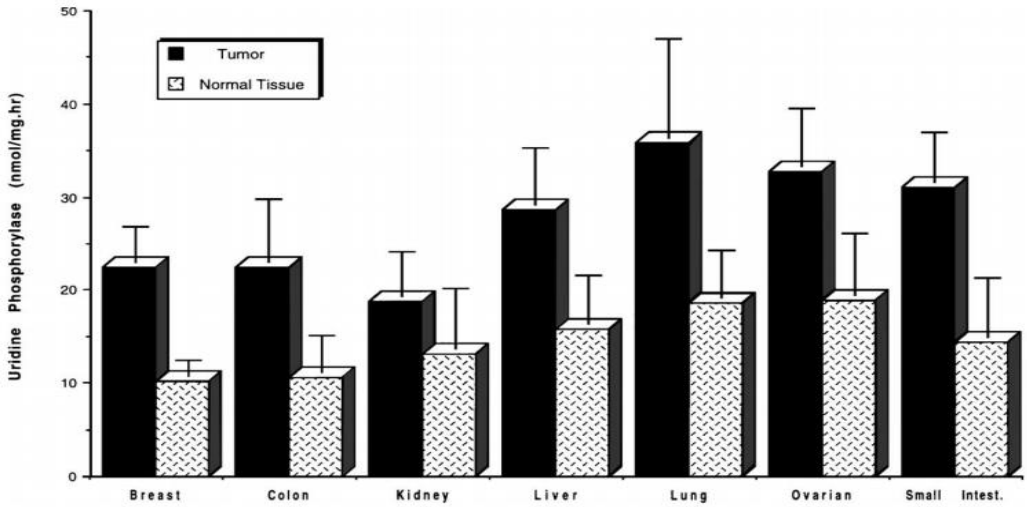


Figure 4. Comparison of UPP levels in tumor tissue with surrounding normal tissue
Reprinted with permission from Pizzorno G.; et al. Homeostatic control of uridine and the role of uridine phosphorylase: a biological and clinical update. *Biochimica et Biophysica Acta* **2002**, *1587*, 133-144.

UPP catalyzes the conversion of 5-FU to 5-flourouridine, which then is converted into several other active metabolites through subsequent downstream reactions.[8] This leads to the formation of multiple downstream metabolites, and it is these processed metabolites that are actually responsible for promoting cell death (Figure 3). Hence, tumor cells are more susceptible to this mechanism of apoptosis due to their increased levels of UPP (Figure 4). The problem with this treatment is that it can cause host toxicity. Rapidly dividing cells that occur naturally in the body, such as those lining the digestive tract, are also prone to accepting these fluorine containing decoys and therefore apoptosis may be induced in normal healthy cells. The major limitation factor with this medication is the inability to raise the dosage amount, if needed, without inducing host toxicity.[6] Recent research has shown natural uridine to be a promising biochemical modulator when combined with 5-FU. The nucleoside ameliorates the toxic effects of 5-FU without any decline in the anti-tumor activity, thereby allowing higher therapeutic doses of 5-FU to be administered for recalcitrant tumors without the negative side effects. It has also been shown that high levels of uridine will selectively relieve 5-FU toxicity in normal tissues, but not tumors.[6] Unfortunately, the same enzyme responsible for one of the two primary pathways by which 5-FU is converted to 5-flourouridine, UPP, also converts uridine to uracil.[8] 5-FU may also be converted to 5-flourouridine through activation by the enzyme orotate phosphoribosyltransferase (OPRT), as shown in Figure 3.

The key to improving this form of cancer therapy is to achieve the perfect balance between high levels of uridine and high levels of 5-FU metabolites by modulating the activity of UPP. However, this is made difficult because of the natural tendency of the

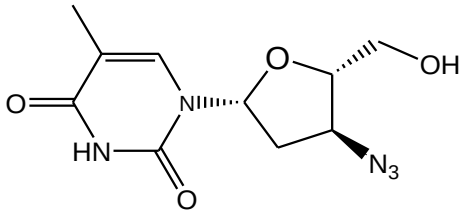
enzyme to move both reactions towards equilibrium, favoring the formation of uracil and 5-flourouridine. If an inhibitor is used to block the conversion of uridine to uracil with the intent of raising uridine levels, it is also effectively blocking a primary pathway for formation of 5-FU active metabolites. This dilemma has sparked an interest to learn as much as possible about UPP in hopes of determining a method for selective inhibition. Elucidation of a purification scheme for the enzyme will provide means for obtaining a pure protein and is the first step to the development of more potent and specific inhibitors.[8,9]

1.1.2 UPP inhibitors in antiretroviral therapy (thymidine analogs)

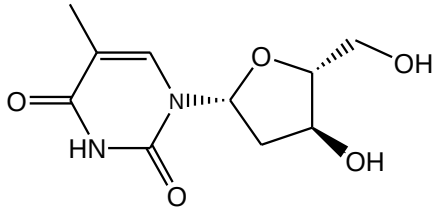
Just as UPP may be targeted to halt the progression of tumor growth, it also shows promise for improving treatment therapies for retroviral infections, such as the deadly and increasingly prevalent human immunodeficiency virus, HIV.[10,11] Since the United States saw its first reported case in June of 1981 of what would later be known as HIV, the HIV/AIDS epidemic has continued to spread. Today, an estimated 1.8 million people have been infected with the virus and 650,000 related deaths have been reported.[12] Due to the impact of the disease both nationally and globally, improvements in treatment options available are critical.

AZT (3'-azido-3'-deoxythymidine) is a thymidine analog antiretroviral drug approved by the FDA for the treatment of HIV/AIDS on March 20, 1987. Although it was first synthesized in 1964 as a potential anti-cancer drug, it was found to be ineffective for that purpose. However, it is effective against HIV by targeting its replication process.[10] Reverse transcriptase replicates HIV RNA by using the nucleotides of the host cell. AZT is structurally very similar to natural thymidine, and therefore

mistakenly becomes incorporated into the new viral DNA strand. Once this occurs, reverse transcription is stopped.[10]



AZT



thymidine

Figure 5. Comparison of AZT and thymidine

Although this drug represented a breakthrough in HIV/AIDS therapy, a similar issue arises with both AZT and the uridine analog anti-cancer drug 5-FU. Effective therapeutic dosage is difficult to achieve due to host toxicity. In the case of AZT, the major limiting factor is hematological toxicity leading to anemia and granulocytopenia.[13] Even when AZT is administered in the highest dose tolerated by the patient, it is not strong enough to stop HIV replication entirely. As of now, this treatment may only slow viral replication and delay HIV progression. Fortunately, UPP has shown promise as a target enzyme for an adjunct treatment that would allow for higher doses of AZT to be administered without the associated side effects. In a study conducted by the Department

of Pharmacology and Comprehensive Cancer Center at the University of Alabama at Birmingham, uridine was found to both selectively protect human bone marrow progenitor cells and reverse the cytotoxic effects caused by AZT.[10] Thymidine showed similar results, however it strongly antagonized the therapeutic effects of AZT while uridine did not interfere with the ability of the drug to inhibit HIV replication.[10]

1.2 Established purification schemes of UPP from various organisms

Determining a purification scheme is a critical initial step in the process of understanding the basic function of an enzyme and how it may be used to advance medicine. UPP has been successfully purified from a number of mammalian tissue sources. This section will detail three of them.

1.2.1 UPP isolated from mouse ehrlich ascites carcinoma cells

Krenitsky *et. al* (Sloan-Kettering Institute for Cancer Research at Cornell University Medical College in New York, New York) have successfully isolated UPP from ehrlich ascites carcimona cells of female adult swiss albino mice.[14] To obtain starting tissue, 0.1 mL of ehrlich ascites tumor cells were inoculated into the peritoneal cavity of 300 mice. After 10 days, the tumors were harvested and prepared in a hypotonic suspension consisting of 0.8% sodium chloride containing 0.2 mg heparin per 50 mL. The solution was homogenized and centrifuged at 10 °C. However all subsequent operations (detailed in Table 1) were performed at 0-4 °C. The method resulted in a 266.73-purification fold with a 2.65% final yield of UPP.[14]

Table 1. **Summary of purification schemes and results**

Source of UPP:	**Mouse ehrlich ascites carcimona cell line[a]**	**Mouse colorectal carcinoma cell line colon-26[b]**	**Rat liver[c]**
Purification scheme:	1.Ammonium sulfate fractionation ↓ 2. DEAE-cellulose column (3.5 x 50 cm) ↓ 3. DEAE-cellulose column (1 x 60 cm) ↓ 4. DEAE-cellulose column (1 x 50 cm)	1. Ammonium sulfate fractionation ↓ 2. DEAE-Toyopearl (18-110 mm) ↓ 3. TSK-G3000sw (7.5 x 600 mm) ↓ 4. TSK-DEAE-5PW (6 x 70 mm)	1. Ammonium sulfate fractionation ↓ 2. Heat ↓ 3. DEAE-Sephadex (2.5 x 90 cm) 2x ↓ 4. DEAE-Sephadex (2.5 x 45 cm) ↓ 5. Sephadex G-200 (2.5 x 90 cm) ↓ 6. Hydroxylapatite (0.9 x 60 cm)
Purification fold:	266.73	10,300	1,894.74
Final UPP yield:	2.65%	23.9%	0.08%

References: **a)** Krenitsky T.A.; Barclay M.; Jacquez J.A. Specificity of Mouse Uridine Phosphorylase: chromatography, purification, and properties. *J. Biol. Chem.* **1964,** *239*, 805-812. **b)** Purification, Cloning , and Expression of Murine Uridine Phosphorylase. *J. Biol. Chem.* **1995,** *270,* 12191-12196. **c)** Cytoplasmic Uridine Phosphorylase of Rat Liver: Characterization and Kinetics. *J. Biol. Chem.* **1971,** *246*, 2021-2030.

1.2.2 UPP isolated from mouse colorectal carcinoma cell line, colon-26

Kraut *et al.* (Department of Biochemistry at the University of Manitoba in Winnipeg, Canada) have isolated UPP from mouse tumor tissue as well, although in this case, the source was colorectal carcinoma cell line, colon-26.[15] To obtain starting tissue, subcutaneous tumor growth was initiated into each of 100 mice by inoculation of 1×10^6 colon-26 cells. After 20 days, the mice were sacrificed and a total of 81.5 g of tumor tissue collected, cut into pieces with surgical scissors, then homogenized at 4° C in 240 mL of a buffer solution consisting of 20 mM potassium phosphate (pH 7.4), 5 mM 2-mercaptoethanol, and 1 mM EDTA. The subsequent purification steps are listed in Table 1, and all purification steps occurred at 4°C. The method resulted in a 10,300-fold purification with a 23.9% yield of pure UPP.[15]

1.2.3 UPP isolated from the cytoplasm of rat liver cells

Also established by Kraut *et. al.* is a purification scheme of UPP from rat liver cytoplasm.[16] For this purification process, the livers of 50 male Holtzman rats were perfused *in situ* with with 0.9% sodium chloride, removed, then homogenized at 4°C with a solution of 0.25 M sucrose, 5 mM **β**-mercaptoethanol. The homogenate was then filtered through four layers of cotton gauze, centrifuged, and the supernatant collected. This and all subsequent steps listed in Table 1 were carried out at 4°C. The process led to a total final yield of 0.08% UPP with a 1,894.74 purification fold.[16]

1.3 Substrate specificity of UPP from varying sources

Obtaining a clear and comprehensive understanding of the substrate specificity of tge enzyme across various organisms significantly aids in the design of effective enzyme inhibitors used to fight disease. The substrate specificity for UPP has been more extensively studies in lower-level organisms because of its longstanding role as a target for anti-parasitics. The benefits of inhibiting the enzyme in higher-level mammals has been a more recent discovery, therefore substrate specificity data for this category are less comprehensive. Furthermore, UPP substrate specificity varies not only among species, but also among tissues and location in the cell.[17] This in combination with variation in technique has caused the information presented in the literature by various authors to appear confusing and at times contradictory. Along with developing a purification scheme for UPP isolated from bovine liver, this thesis will clarify the substrate specificity of the enzyme isolated from this particular source with regard to inosine and thymidine. Listed below are substrate specificities for UPP isolated from a variety of sources (Table 2). The information was originally compiled and presented by Schromburg in his book *Class 2- Transferases VI* of the Springer Handbook of enzymes series.[18]

Table 2. Comparison of UPP substrate specificity across various organisms [26-52]

Organism:	Substrate:	Activity ratio:	References:
Prokaryotes			
Escherichia.coli			
	uridine	100	19-24
	thymidine	2	
	2'-deoxyuridine	6	
	5-bromo-2'-deoxyuridine	27	
	5-bromourdine	69	
	5-flouro-2'-deoxyuridine	14	
	thymine ribonucleoside	19	
Haemophilus influenza			
	uridine	100	25
	thymidine	21	
	2'-deoxyuridine	12	
	5-bromo-2'-deoxyuridine	75	
	5-bromouridine	40	
	5-methyluridine	27	
	uracil arabinoside	10	
Lactobacillus casei			
	uridine	100	26,27
	thymidine	*ND	
	5-bromouridine	40	
	5-methyluridine	*ND	
Enterobacter aerogenes			
	uridine	100	28
	thymidine	22	
	2'-deoxyuridine	18	
	uracil arabinoside	10	
Acholeplasma laidlawii			
	uridine	100	29

Table 2 cont. Comparison of UPP substrate specificity across various organisms [26-52]

Eukaryotes			
Giardia Lamblia			
	uridine	100	30
	thymidine	100	
	2'-deoxyuridine	100	
a single enzyme may be responsible for the conversion of all three substrates			
Hymenolepis diminuta			
	uridine	100	31
	thymidine	*ND	
Salmonella typhimurium			
	uridine	100	32
	2'-deoxyuridine	*ND	
	5-bromouridine	*ND	
	5-flourouridine	*ND	
Dictyostelium discoideum			
	uridine	100	33
Schistosoma mansoni			
	uridine	100	34
	thymidine	*ND	
	2'-deoxyuridine	*ND	
Mammals			
Mus musculus			
	uridine	100	8,17,35-40
	thymidine (liver)	5	
	2'-deoxyuridine	*ND	
	5'-deoxy-5-flourouridine	25	
	5-bromouracil		
	5-fluoro-2'-deoxyuridine	15	
	5'-fluorouridine	85	
	5-methyluridine	*ND	
	azathymine	*ND	
	azauracil	*ND	
	thymine	*ND	

Table 2 cont. Comparison of UPP substrate specificity across various organisms [26-52]

Rattus norvegicus			
	uridine	100	41-46
	thymidine	*ND	
	2'-deoxyuridine	cytosolic enzyme shows activity while plasma membrane enzyme activity is minimal to none.[53]	
	arabinofuranosyl-5-ethyluracil	weak substrate	
Homo sapiens			
	uridine	100	8,17, 36,37,39,47
	thymidine (liver)	0	
	thymidine (placenta)	2	
	2'-deoxyuridine	*ND	
	5-flourouridine (liver)	15	
	5-flourouridine (placenta)	70	

*ND indicates that the the nuceloside has been identified as a substrate; however, no value has been determined for the substrate specificity.

Information originally compiled by: Schomburg, D., Schomburg, I., & Chang, A. Uridine Phosphorylase. *Class 2 -Transferases VI*. Springer Handbook of Enzymes. Burlin: Springer. **2007**, *33*, 39-51.

1.4 Enzyme structure

Uridine phosphorylase clearly shows promise as a target for the development of new drugs (*e.g.* antibiotics, antivirals, and modulators for chemotherapeutic agents). In order to develop these drugs, a clear and detailed understanding of the enzyme in its native form, transition state, and in complex with low-molecular compounds must be obtained. In addition, information on how the enzyme differs structurally, functionally, and metabolically among organisms and while in complex with various substrates is critical for developing selective inhibitors. Because the enzyme has been successfully purified from several organisms, much information has been obtained about the structure and function of the enzyme as it varies across organisms.

1.4.1 UPP isolated from E.coli

An initial understanding of UPP and its mechanism of catalysis have been obtained through an in-depth study of the enzyme isolated from *E.coli*. The substrate specificity in primitive microorganisms is usually much lower than that of more developed species, meaning that UPP will often accept uridine, thymidine, or cytidine as a substrate, although it typically favors uridine. In mammals, UPP shows a much stronger affinity for uridine than thymidine, and a separate thymidine phosphorylase enzyme is relied on for the salvaging of thymine.[48] This is a key feature to developing antimicrobials and anti-parasitics, as disabling the UPP enzyme in these organisms will slow or block their ability to recover thymine in the nucleoside salvage pathway. UPP in all organisms completely excludes purines as substrates due to a small binding pocket at the specificity region. Bacterial UPP exists as a hexameric, trimer of dimers, with each homologous subunit being formed of 253 amino acids with a total mass of 27 kDa.[2] Each

dimer contains two monomers (Fig. 6c) with separate active sites, but each active site contains three residues (Phe7, His8, and Arg48) which are donated by the other monomer. Therefore the smallest functional unit is the dimer (Figure 6b). Hydrophobic residues are responsible for most of the interactions that hold the dimers together to make a hexameric structure (Fig. 6a).

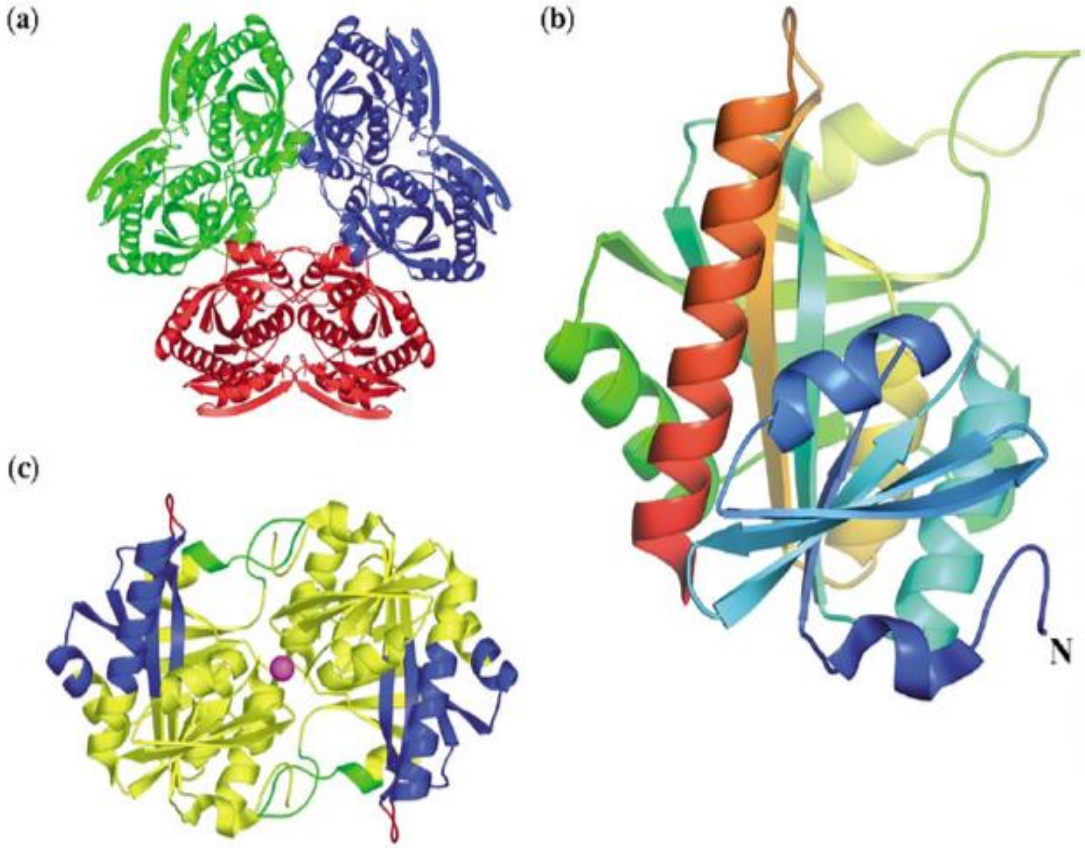


Figure 6. Uridine phosphorylase isolated from *E.coli* (a) Hexameric trimer of dimers structure. (b) UPP monomer composed of a mixed B sheet core imbedded in a network of a-helices. (c) Dimer structure with central phosphate atom (magenta) Reprinted with permission from: Caradoc-Davies TT, Cutfield SM, Lamont IL, Cutfield JF. Crystal structures of *Escherichia coli* uridine phosphorylase in two native and three complexed forms reveal basis of substrate specificity, induced conformational changes and influence of potassium. *J. Mol. Biol*. **2004,** *337,* 337-354.

1.4.2 UPP in higher level organisms

UPP has progressively been reduced in size and number of subunits with the evolution of more complex species. A tetrameric form exists in many mammals including rat, while human and bovine exhibit a dimeric structure (Figure 7). Two homologs exist in most upper level organisms, UPP1 and UPP2, although UPP2 appears to be sporadically disappearing from many organisms and, in humans, it is predominately limited to kidney tissues.[49] It is thought that the two homologs came about as an early evolutionary event and UPP1 plays a more active role in the activation of fluorinated pyrimidine nucleoside analogs.[4]

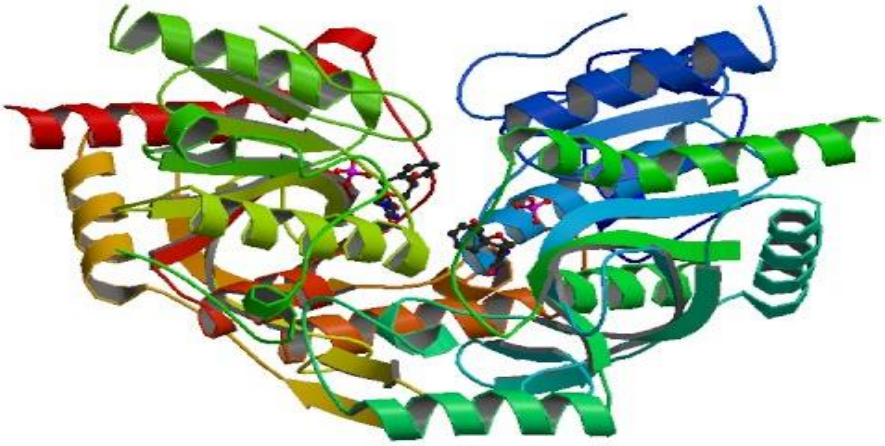


Figure 7. Human uridine phosphorylase II (recombinant) in complex with benzylacyclouridine Reprinted with permission from the Protein Data Bank PDB ID: 3POE Rooslid, T.P. Castronovo, S. Villoso, A. (2011) A novel structural mechanism for redox regulation of UPP2 activity. J. Struct. Biol. 176. 229-237.

1.5 Active site

The active site of the enzyme is largely conserved for UPPs of all organisms and is contained within a cavity composed of three binding sites; one for phosphate, one for ribose, and one for uracil (Figure 8).

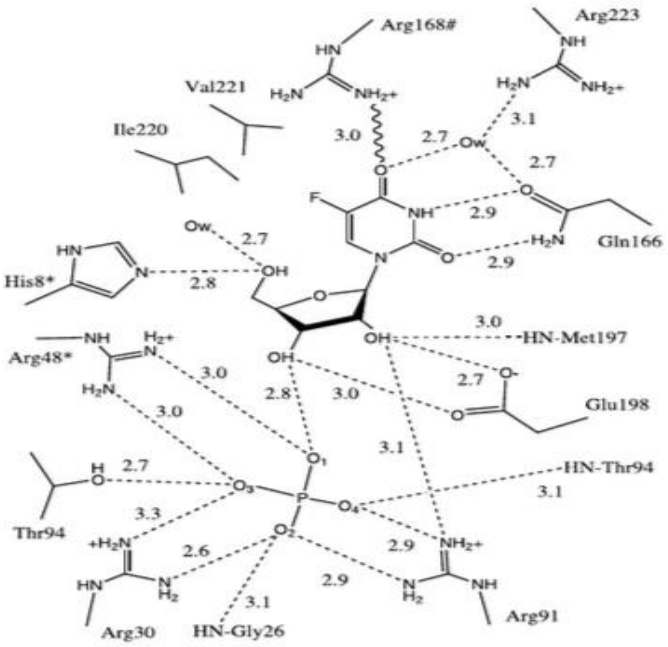


Figure 8. Active site of UPP A network of hydrogen bonds holds the ribose, base, and phosphate group in place. * indicate that the residue originates from the neighboring monomer. # indicate a weak hydrogen bond interaction. Source: Caradoc-Davies TT, Cutfield SM, Lamont IL, Cutfield JF. (2004). Crystal structures of *Escherichia coli* uridine phosphorylase in two native and three complexed forms reveal basis of substrate specificity, induced conformational changes and influence of potassium. The wavy line between Arg 168 and O4 of uracil indicates an unfavorable geometry for hydrogen bond formation. Reprinted with permission from: Caradoc-Davies TT, Cutfield SM, Lamont IL, Cutfield JF. Crystal structures of Escherichia coli uridine phosphorylase in two native and three complexed forms reveal basis of substrate specificity, induced conformational changes and influence of potassium. *J. Mol. Biol*. **2004,** *337,* 337-354.

1.5.1 Uracil binding site

Uracil or uracil analogs, such as 5-FU, are stabilized in the active site by a network of hydrogen bonds created by the residues Arg168, Gln166, Met197, and a deeply buried water molecule. These three residues are conserved among all UPPs, from the early prokaryotic enzyme to the highly developed eukaryotic form. They play a crucial role in enzyme specificity for uridine and to a much lesser extent, the other pyrimidines. The O4 atom of uracil or its analogs form hydrogen bonds with the nitrogen atom of Arg168, and to Arg223 and the carbonyl oxygen of Gln166 through a water molecule. Gln166 also forms hydrogen bonds directly to the O2 and N3 atoms of uracil, and is key in forming the discriminating interaction with uracil[2,50] (Figure 8).

1.5.2 Ribose binding site

The ribose binding site is located between the phosphate and uracil binding sites and is formed by the residues Arg91, Met197, and Glu198 from the monomer containing the active site and His 8 from the neighboring subunit.[2] These residues are characteristic of the ribose binding site in all UPP from varying sources. Met197 and Arg 91 form hydrogen bonds with the 2' hydroxyl group of the ribose moiety and Glu198 forms a pair of stabilizing hydrogen bonds bidentate to the 2' and 3' hydroxyl groups. The 5' hydroxyl forms hydrogen bonds with the nitrogen atom of His 8 and with a bridging water molecule[2,50] (Figure 8).

1.5.3 Phosphate binding site

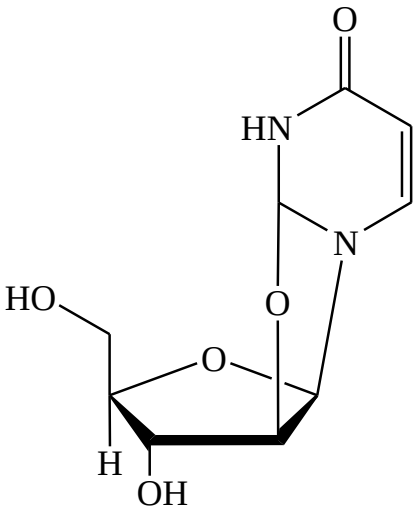
The phosphate binding site is located directly below the ribose binding site. It is occupied by either phosphate ion or phosphate in complex with ribose-1-

phosphate.[50] In the case of the latter, the O1 molecule of phosphate forms a connecting hydrogen bond to the 3' hydroxyl group of ribose. The binding pocket is lined by three residues: Arg30 and Arg90 (from the monomer containing the active site) and Arg48 (contributed from the neighboring subunit). Each residue forms hydrogen bonds bidentate to two oxygen atoms of the phosphate group. Thr94 also hydrogen bonds to phosphate in two places. The main chain bonds to the O4 atom while the side chain hydrogen bonds to the O3 atom. Additionally, the O2 atom forms a hydrogen bond to the main chain of Gly26[2,50] (Figure 8).

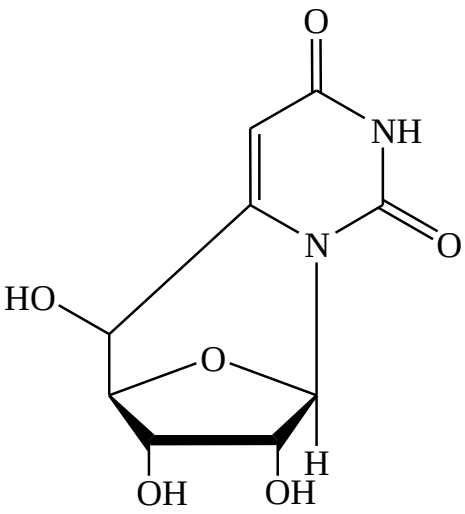
1.6 Existing inhibitors of UPP

Elucidation of the residues and binding interactions of the active site has made the development of several types of UPP inhibitors possible. To date, all UPP inhibitors under study can be divided into four main groups.[2,51] They are classified based on the conformation of the uracil ring with respect to the N-glycosidic bond.[2,51] The classes are listed below, and the structure of an example from each class is given in Figure 9.

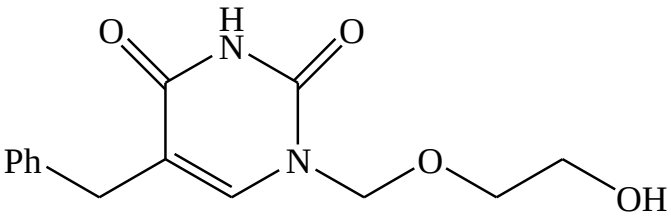
1. Fixed in the *syn* conformation (ex. 2,2'-anhydrouridine)[2,51]
2. Fixed in the *anti* conformation (ex. 6,5'-cyclouridine)[2,51]
3. Non-fixed (ex. benzylacyclouridine of the acyclouridine class)[2,51]
4. Without N-glycosidic bond (ex. Carbocyclic compounds)[2,51]



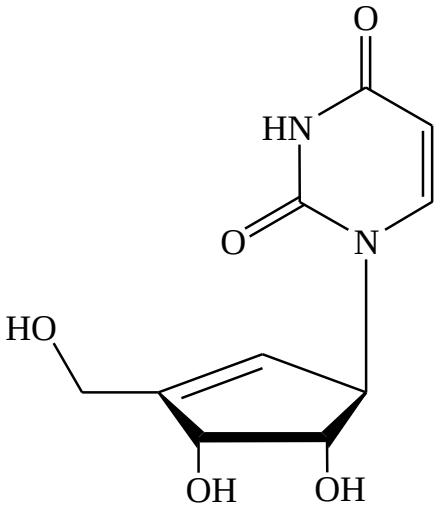
2,2'-anhydrouridine



6,5'-cyclouridine



5-benzylacyclouridine



carbocyclic uridine

Figure 9. An example from each of the four classes of inhibitors currently under study

In a study conducted by el Kouni and coworkers, it was found that the 2,2'-anhydrouridines bound most tightly to UPP and thus resulted in the greatest degree of inhibition, followed by the acyclouridine class.[51] The 6,5' cyclouridines and the carbocyclic compounds failed entirely to bond with UPP and thus exhibited no inhibition. Due to the results of this study, el Kouni and coworkers suggest that inhibitors bind to UPP as *syn*-rotamers. Acyclouridines also bind tightly to UPP behind 2,2'-anhydrouridines because they have free rotation about the N-glycosidic bond and are able to rotate to the *syn* conformation. The lack of bonding of the carbocyclic compounds suggest that a true N-glycosidic bond is necessary for an inhibitor to bind to UPP.[51]

As an anticancer adjunct therapy to 5-FU, the use of inhibitors have proven to raise plasma uridine levels (and therefore reduce host toxicity of 5-FU) by competitively inhibiting UPP and forcing 5-FU to be activated by OPRT.[52,53] Although this mechanism of action has been effective, it has a disadvantage in that it causes one of the major activation pathways of 5-FU to be blocked.

5-Benzylacylclouridine (BAU) derivatives, a member of the acyclouridine class, are a widely studied class of inhibitors that have been used in conjunction with 5-FU. Although overall successful in lowering host toxicity of 5-FU, a negative feature of BAU's is that they have low water solubility, which poses a concern of crystallization in the bladder.[54,55] Also, the more potent derivatives have been shown to induce high toxicity.[54]

1.7 Discussion/ thesis goals

The inhibition of UPP has proven to have a wide variety of applications in medicine. Natural uridine has shown a promising ability to rescue normal tissues and, in some cases, even reverse the cytotoxic effects associated with anti-retroviral and anti-cancer chemotherapy. Additionally, the differences that exist between the early prokaryotic form of the enzyme and the dimer form seen in higher ordered animals can be exploited to design inhibitors that would serve as antibiotics and antiparasitics.[2] The goal of my thesis research is to determine a purification scheme for isolating UPP from bovine liver, as bovine liver is readily available and 87% similar to the human isoform.[9] Once the enzyme has been purified, the substrate specificity with regard to thymidine and inosine will be determined. This purification scheme and substrate specificity information will serve as a platform for several avenues of future work, including determination of the transition state. Enzymes bind most tightly to their substrates during the transition state; therefore, analogs that resemble the transition state would be expected to bind more tightly than the natural substrate and would serve as potent competitive inhibitors. The application of this acquired knowledge is twofold: first, to allow for improved effectiveness and lower toxicity of existing inhibitors and second, to aid in the development of a new class of inhibitors that would be able to selectively inhibit UPP in various reactions.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Bovine liver was purchased from C and F Meats of Triune, TN. Uridine, uracil, inosine, hypoxanthine, thymidine, thymine, and protease inhibitor cocktail for mammalian cells and tissue were purchased from Sigma Aldrich. A stirred ultrafiltration cell was purchased from Amicon. Ultrafiltration membranes were also purchased from Amicon. HiLoad Sepharose Mono Q Fast Flow (16/10) ion exchange column and Sephacryl S100 (26/60) size exclusion column for the AKTA purifier system were purchased from GE Healthcare. Bio-scale CHT-5-I Ceramic Hydroxyapatite column and protein dye were purchased from Bio-Rad. All other chemicals used were reagent grade.

2.2 Methods

2.2.1 Activity assays

After each step in the purification process, nucleoside phosphorylase activities for UPP, thymidine phosphorylase (TPP), and inosine phosphorylase (IPP) were measured by the disappearance of nucleoside and appearance of the respective base by HPLC analysis, or by appearance of ribose by reducing sugar assay.

The HPLC assay reaction mixture was 1 mL in total volume, and consisted of nucleoside (1 mM) and sodium arsenate (10 mM) at a pH of 7.2. Initiation of the reaction occurred with the addition of 100 μL of enzyme extract. The sample was immediately assayed over the course of several hours with an injection volume of 10 μL at 30-60 minute intervals on a Dionex UltiMate 3000 HPLC equipped with a UV

detector. The nucleoside and base were separated on a Phenomenex Hyperclone ODS C_{18} HPLC column (150 x 4.6 mm, 5 μM particle size). Substrates and products were eluted isocratically with a mobile phase of 90% 10 mM ammonium phosphate (pH 5.2) and 10% methanol. Both substrates and products were detected at 254 nm. Standard samples for each nucleoside and base were run prior to every set of sample assays to establish retention times. The disappearance of nucleoside was used to determine the enzyme activity by plotting the area under the peak against time.

Additionally, a reducing sugar assay was used to assay for activity of UPP, TPP, and IPP.[58] The reducing sugar reaction mixture consisted of nucleoside (1 mM), sodium arsenate (10 mM) pH 7.2 and 100 μL of each enzyme fraction. A control containing the same nucleoside (1 mM) and sodium arsenate (10 mM) pH 7.2 without enzyme present was also analyzed. Reaction mixtures were incubated for 24 hours at 37°C. After incubation, 300 μL of copper sulfate reagent and 300 μL of neocuproine reagent were added to each fraction. The fractions were then incubated at 100°C for 7 minutes and the absorbance was read at 450 nm for each fraction. Fractions containing the highest enzyme activity were pooled.

2.2.3 Protein concentration

Protein concentration was determined by two methods: absorbance at 280 nm and Bio-Rad Protein dye method. For those samples whose absorbance was determined at 280 nm, the amount of protein present can be estimated by assuming that a 1mg/ml protein solution will have an absorbance reading of 1.3.[59] The following formula, protein concentration (mg/mL) = $1.3A_{280}$, can then be used to calculate protein concentration.[56.]

Using the Bio-Rad dye method, each sample of unknown protein content was serially diluted 1:10, 1:100, and 1:1000 times. Then in separate test tubes, 10 μL of each dilution were combined with 790 μL of distilled water and 200 μL of Bio-Rad dye. The samples were vortexed and assayed at 595 nm using a Hitachi U-200 spectrophotometer. A standard curve was made by graphing concentrations of bovine serum albumin (1.44 ug/mL) against corresponding absorbencies before each assay. The absorbance readings of unknown samples were compared to the standard curve to determine the protein concentration (Fig. 10).

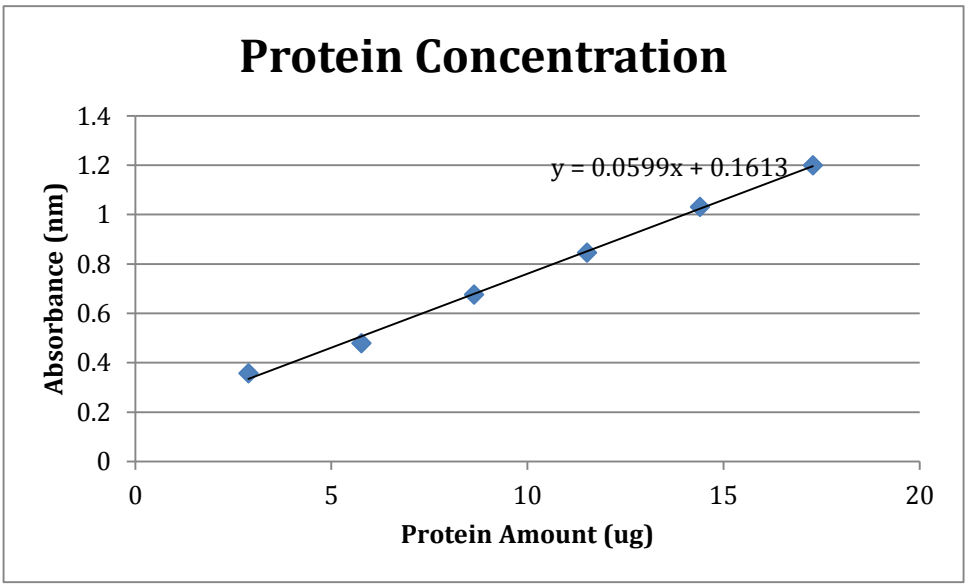


Figure 10. Standard curve relating protein amount to absorbance

2.2.4 Preparation of bovine liver initial extract

Bovine liver (20 g) was cut into half inch pieces and placed in 100 mL of 10 mM sodium phosphate buffer pH 7.2. Dithiothreitol (12 mg) was added to protect against oxidation, along with 50 μL of protease inhibitor cocktail. Protamine sulfate (1 g) was added to bind DNA and cause it to precipitate. The mixture was homogenized at 4º C for a total of six minutes (six one minute intervals with one minute rest between blends) to lyse the bovine liver cells, releasing cytosolic UPP. The sample was centrifuged at 15,000 *xg* for 30 minutes at 4º C and the supernatant (100 mL) was collected.

2.2.5 Purification scheme

The supernatant collected after centrifugation of the initial extract from the bovine liver was treated with 17.6 g of ammonium sulfate, bringing the sample to 30% saturation. The supernatant was placed on ice, and the ammonium sulfate was added slowly while stirring over the course of 15 minutes. The solution was covered with foil, and stored at 4°C for 12 hours. The sample was then centrifuged at 15,000 *xg* for 30 minutes and the supernatant collected. Next, the sample was brought to 60% saturation by addition of another 19.5 g of ammonium sulfate, using the procedure described above. After centrifugation, the supernatant was discarded and the pellet collected. The pellet was resuspended in 10 mM sodium phosphate buffer (pH 7.2) to a total volume of 40 mL. Samples were taken from both the supernatant at 30% saturation and the resuspended pellet at 60% saturation and assayed for activity by the methods described.

The resuspended pellet was dialyzed against 2 L of 10 mM sodium phosphate buffer pH 7.2 for two days, with the buffer being changed once. The dialyzed sample was then concentrated from 40 mL to 10 mL using an Amicon 8050 stirred ultrafiltration cell. The concentrated sample was loaded onto a HiLoad Sepharose Fast Flow Mono Q column (16/10) attached to an FPLC system. The column was washed with 40 mL of 10 mM potassium phosphate buffer pH 7.2 followed by 400 mL of a linear gradient of potassium phosphate buffer from 10 mM to 800 mM. Fractions (10 mL) were collected at a flow rate of 2 mL/min and assayed for protein content by measuring the absorbance at 280 nm and for activity against UPP, TPP, and IPP by reducing sugar assay. Fractions containing UPP activity were pooled, concentrated from 30 mL to 8 mL again by ultrafiltration, and assayed again for UPP activity by HPLC analysis. The sample was then split into a 5 mL sample and a 3 mL sample.

The first sample (5 mL) was loaded onto Sephacryl S100 (26/60) size exclusion FPLC column. The mobile phase used was 300 mM sodium chloride in 100 mM potassium phosphate pH 7.2 buffer. The column was eluted with a total of 637 mL isocratically at a flow rate of 1 mL/ min and 10 mL fractions were collected. The fractions were assayed for activity by the reducing sugar method outlined previously. The process was repeated for the second sample (3 mL) and the fractions that showed UPP activity from each batch were pooled, dialyzed, concentrated down to 7 mL, then assayed again for UPP activity by HPLC analysis.

The sample was then split into 2 batches and the first batch (4 mL) was loaded onto a Bio-Rad CHI Hydroxyapatite column. The column was washed with 30 mL of

10 mM potassium phosphate buffer pH 7.2 followed by 100 mL of a linear gradient of potassium phosphate buffer from 10 mM to 800 mM. Fractions (5mL) were collected from at a flow rate of 1 mL/min. The fractions were assayed for UPP activity by reducing sugar assay using the standard procedure. The second batch (3 mL) was loaded onto the column and the same procedure was followed. Fractions containing UPP only were pooled and assayed again for activity against UPP, TPP, and IPP using HPLC analysis.

2.2.6 Substrate specificity

Substrate specificity was determined by comparing the velocities (by the method described) at which UPP converted uridine to uracil, thymidine to thymine, and inosine to hypoxanthine.

CHAPTER III

RESULTS AND DISCUSSION

Each step of the purification process is designed to eliminate unwanted proteins while retaining as much of the target protein, uridine phosphorylase (UPP), as possible. It became apparent during the purification process that UPP is difficult to isolate from two other phosphorylase enzymes: thymidine phosphorylase (TPP) and inosine phosphorylase (IPP) (Figure 11).

3.1 Determination of enzyme activity

In order to track the progression of isolating UPP from the other two proteins, activity for each enzyme was monitored after each column purification step. Two methods exist for detecting enzyme activity. As Figure 11 shows, the nucleoside (uridine, thymidine, or inosine) is catabolized in the presence of phosphate into the corresponding base (uracil, thymine, or hypoxanthine) and ribose-1-phosphate. One method of determining whether a particular phosphorylase is present and catalyzing its reaction is to measure either the disappearance of nucleoside or the appearance of base by HPLC. Table 3 shows the standard retention times for the nucleosides (uridine, thymidine and inosine) and the bases urine, thymine, and hypoxanthine) While this is a highly reliable method, it requires around 30 minutes per sample. Therefore it is not a practical way to assay more than a few samples. Since a typical column produces more than thirty fractions, analysis by HPLC would require almost 15 hours.

Another option for measuring phosphorylase activity is to determine the amount of α ribose-1-phosphate being formed using a phosphate assay.

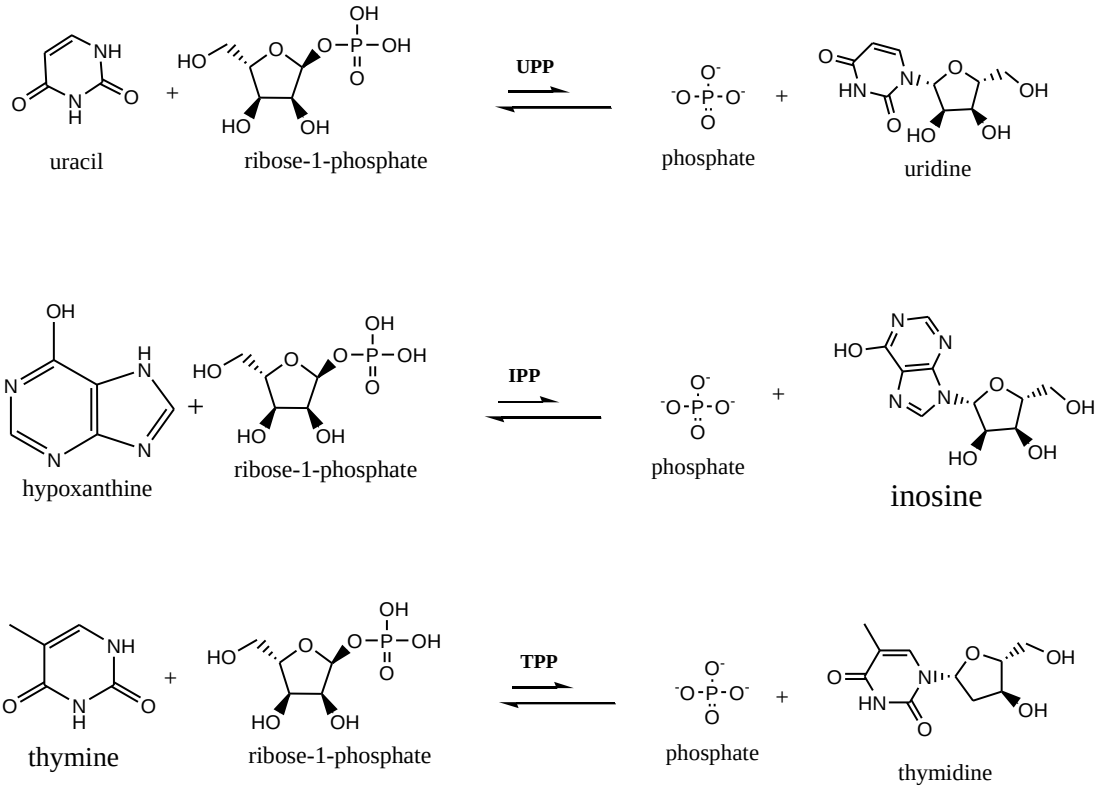


Figure 11. Reactions catalyzed by nucleoside phosphorylases in nucleoside salvage pathways (a) UPP catalyzes the reversible catabolism of uridine into uracil and ribose-1-phosphate in the presence of phosphate. (b) TPP catalyzes the reversible catabolism of thymidine into thymine and ribose-1-phosphate in the presence of phosphate. (c) IPP catalyzes the reversible catabolism of inosine into hypoxanthine and ribose-1-phosphate in the presence of phosphate.

This method has its problems as well because the phosphate buffer used would interfere with the results. However, Parks *et al.* have shown that the phosphorylase reaction will proceed by the same mechanism if arsenate is used *in lieu* of phosphate, yielding α ribose-1-arsenate. Ribose-1-arsenate is unstable and will spontaneously hydrolize into its components, ribose and arsenate.[57] Since ribose is a reducing sugar, a reducing sugar assay can be used to determine the presence of ribose and hence, the activity of the enzyme. To determine the amount of the reducing sugar ribose present in each column fraction, a neocuproine/ copper sulfate assay was used.[58,59]

Table 3. Typical retention times of nucleosides: uridine, thymidine, and inosine, and bases: uracil, thymine, and hypoxanthine

Nucleoside:	**Retention time: (min)**
Uridine	3.7
Thymidine	11.1
Inosine	6.7
Base:	
Uracil	2.5
Thymine	4.5
Hypoxanthine	3.9

Data was collected using a Dionex UltiMate 3000 HPLC equipped with a UV detector and a Phenomenex Hyperclone ODS C_{18} HPLC column (150 x 4.6 mm, 5 μM particle size).

3.2 Purification by ammonium sulfate fractionation

The first step of the purification process is the ammonium sulfate fractionation, a technique that uses differences in solubility to separate proteins.[60] Proteins have hydrophilic amino acid side-chains that interact with water and cause them to be soluble in aqueous media. When ammonium sulfate salts are added, the salts compete for the available water molecules, causing the protein to be less soluble in water. As the salt concentration increases, a point is reached at which the number of water supply molecules accessible to the protein is insufficient for the protein to fully dissolve. At this point, the proteins begin to interact with one another and precipitation occurs. This is a useful first step in protein purification because solubility depends on the ionic strength, which differs markedly among proteins. A protein with lower ionic strength will be less soluble in water and will therefore precipitate at a lower salt concentration than a protein with higher ionic strength.[60]

To precipitate UPP out of the initial bovine liver homogenate, 17.6 g of ammonium sulfate was added, bringing the salt content to 30%. The intention of this step was to cause proteins of lower ionic strength/ lower solubility to form a precipitate while leaving most of the target protein, UPP, still dissolved in solution. However; after centrifugation, only a very small amount of pellet was observed. 17.6 g of ammonium sulfate again added, bringing the salt content up to 60%. This time, after centrifugation, a fair amount of pellet was present. The pellet was then resuspended in 10 mM sodium phosphate buffer to a final volume of 40 mL. Both the supernatant at 30% saturation and the pellet at 60% saturation were assayed for activity by HPLC, and the protein content determined by Bio-Rad assay. At 60% salt

concentration, the total protein dropped from 1176 mg to 523 mg. 62.78% of UPP was recovered, meaning that 37.22% of UPP was still dissolved in the supernatant. This was necessary, as a bulk of highly soluble, unwanted proteins were also dissolved in the supernatant. This step was carried out at 4°C to maintain protein stability, and ultimately led to a purification fold of 1.41

3.3 Purification by Mono Q ion exchange chromatography

After the ammonium sulfate step, the next purification step was ion exchange chromatography using a Mono Q FPLC column eluted with a linear gradient of 10 mM to 800 mM potassium phosphate. Ion exchange columns are useful for the separation of proteins or any other charged molecule based on their difference in charge.[61] The resin of the column may either be positively charged (anion exchange column) or negatively charged (cation exchange column). The Mono Q column used in this purification scheme uses positively charged quaternary amine ligands as the anion exchanger (stationary phase). This causes the negatively charged phosphorylase enzymes to be retained on the column's stationary phase. As the potassium phosphate mobile phase passes through the column, the ions will displace the enzymes as long as they are of stronger ionic strength. As the salt concentration increases along the gradient, enzymes are eluted off into separate fractions and separation is achieved.[61] The resulting fractions were assayed by 280nm absorbance and reducing sugar assay, respectively. The protein elution profile is shown in Figure 12. As can be seen, a significant portion of protein did not bind to the column and eluted in the wash. Protein elution started at around 100 mM potassium phosphate. Proteins continued to elute until just before the maximum salt concentration of 800 mM potassium

phosphate, however the major portion of protein elution was between 100 mM and 600 mM potassium phosphate.

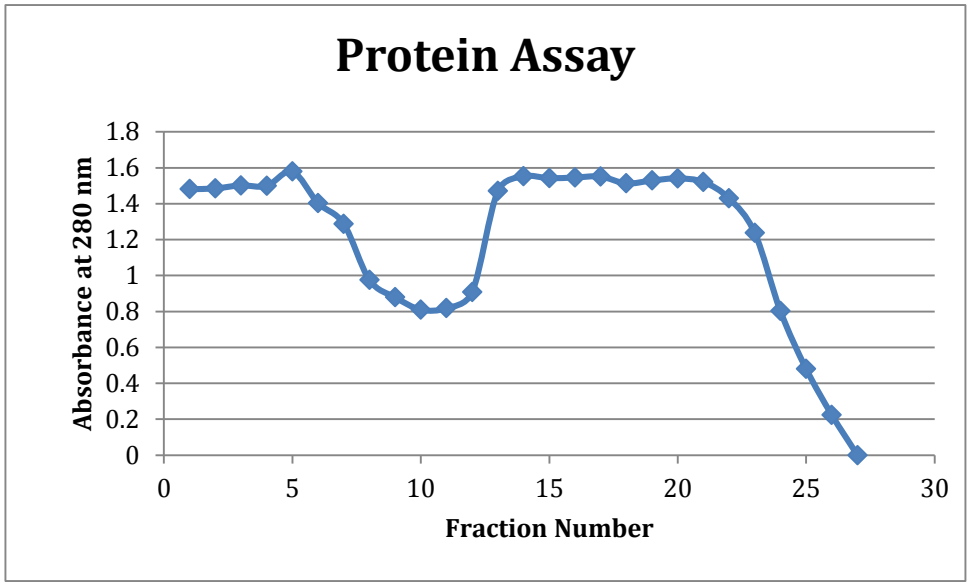


Figure 12. Protein elution profile for Mono Q column

Figure 13 shows the resulting chromatograms from the reducing sugar assay. The ribose levels (and thus the enzyme activities) of UPP (Fig. 13a) TPP (Fig. 13b), and IPP (Fig. 13c) present in each fraction eluted off of the Mono Q column are compared. UPP and TPP showed a similar elution profile. The chromatograms reveal that IPP tends to elute off of the column in a greater number of fractions. This results in a much broader peak (Fig. 13c) and therefore makes it very difficult to obtain a sample of UPP without including higher levels of IPP in the sample. The fractions with the greatest amount of UPP were pooled into a single sample. (Fractions 13-20)

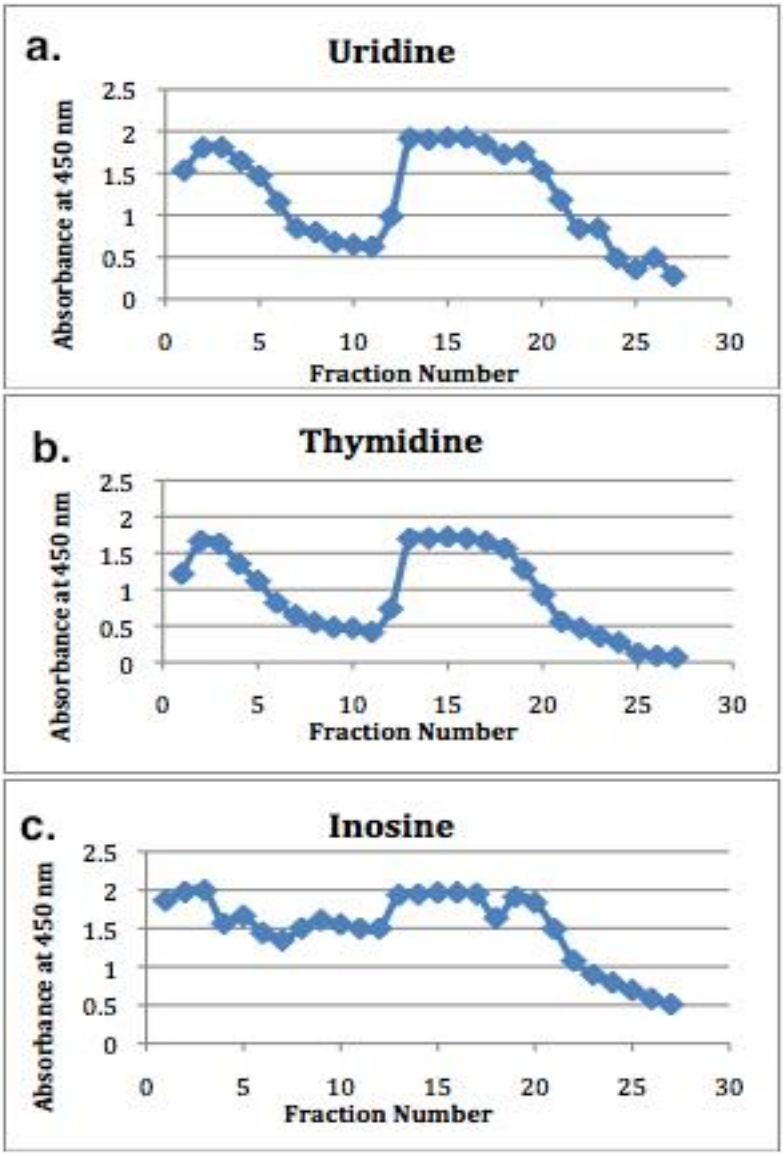


Figure 13. Activity elution profile for UPP, TPP, and IPP from Mono Q column (a) One mL of each fraction is added separate test tubes, each containing a uridine reaction mixture. The chromatogram indicates some UPP content in the wash, Fractions 1-6, and a strong presence of UPP in Fractions 13-20. (b) One mL of each fraction is added to separate test tubes, each containing a thymidine reaction mixture. The chromatogram indicates some TPP content in the wash, Fractions 1-5, and a strong presence of TPP in Fractions 13-19. (c) One mL of each fraction is added to separate test tubes, each containing an inosine reaction mixture. The chromatogram indicates high levels of IPP content in all fractions from 1-20, with slightly higher levels in the wash, Fractions 1-3, and in Fractions 13-20.

3.4 Purification by Sephacryl S100 (26/60) size exclusion chromatography

A size exclusion column was next used to purify UPP. This method separates proteins and other macromolecules based on their difference in molecular size.[62] The stationary phase is composed of porous particles packed tightly into the column as the solid matrix. Initially, a liquid mobile phase buffer containing no protein flows through the column. Next, the same buffer is added to the column, this time containing the proteins to be separated. Unlike the ion exchange chromatography method previously detailed, the proteins will not adsorb to the matrix. The separation for this technique relies solely on the variation in protein size. Molecules that are too large to fit through the pores in the beads will flow through the column fastest and will elute first. Smaller molecules, which enter the pores, will be retained in the column and will have a longer elution time.[62] The Sephacryl S100 used for this procedure has an average particle size of 47μm and was selected for use because it is ideal for separating proteins of molecular weight $1 \times 10^3 - 1 \times 10^5$ Daltons for globular proteins, a range encompassing the molecular weight of many UPP enzymes.[18] Because the volume of the sample loaded can affect the efficiency of the separation, the sample was split into two batches, the first 5 mL and the second 3 mL. The samples were loaded consecutively onto the Sephacryl S100 (26/100) and eluted isocratically with 300 mM sodium chloride in 100 mM potassium phosphate pH 7.2 buffer. Figure 14 shows the protein elution profile measured at 280 nm.

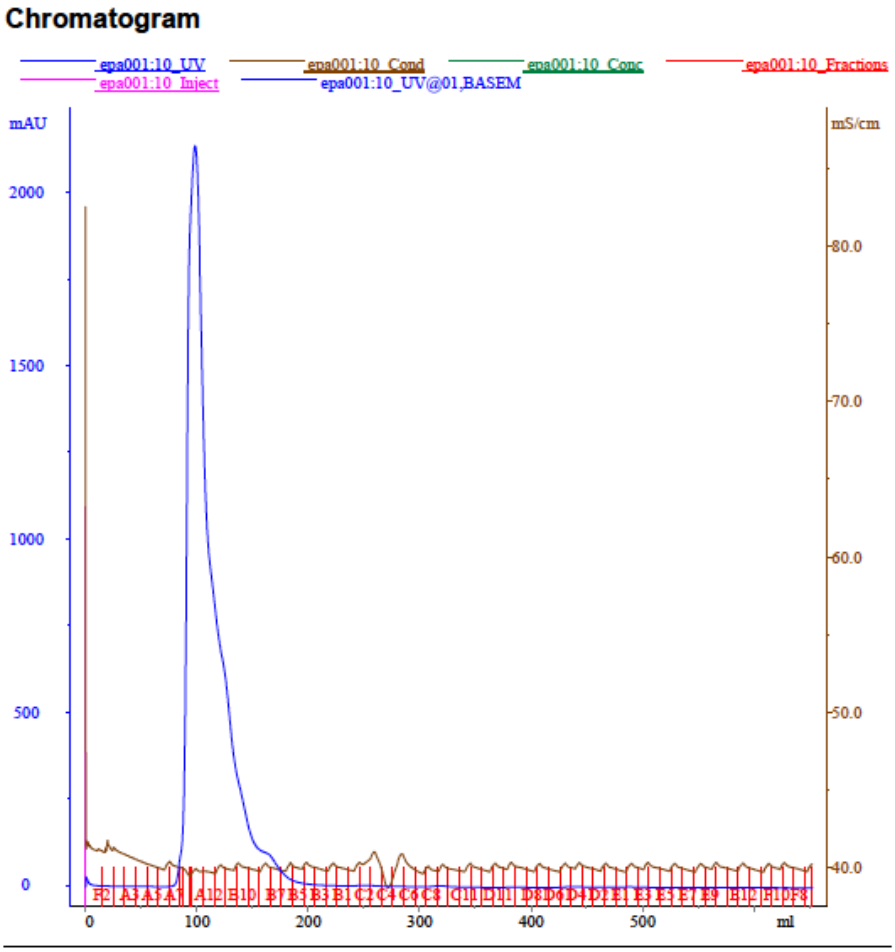


Figure 14. Protein elution profile for Sephacryl S100 size exclusion column

The resulting reducing ribose assay chromatograms for the first and second batches are shown below in Figure 15. Just as with the Mono Q column, the absorbance of ribose was measured at 450 nm for each fraction to determine enzyme activity for UPP (fig. 15a and 15b), TPP (fig. 15c and 15d) and IPP (fig. 15e and 15f).

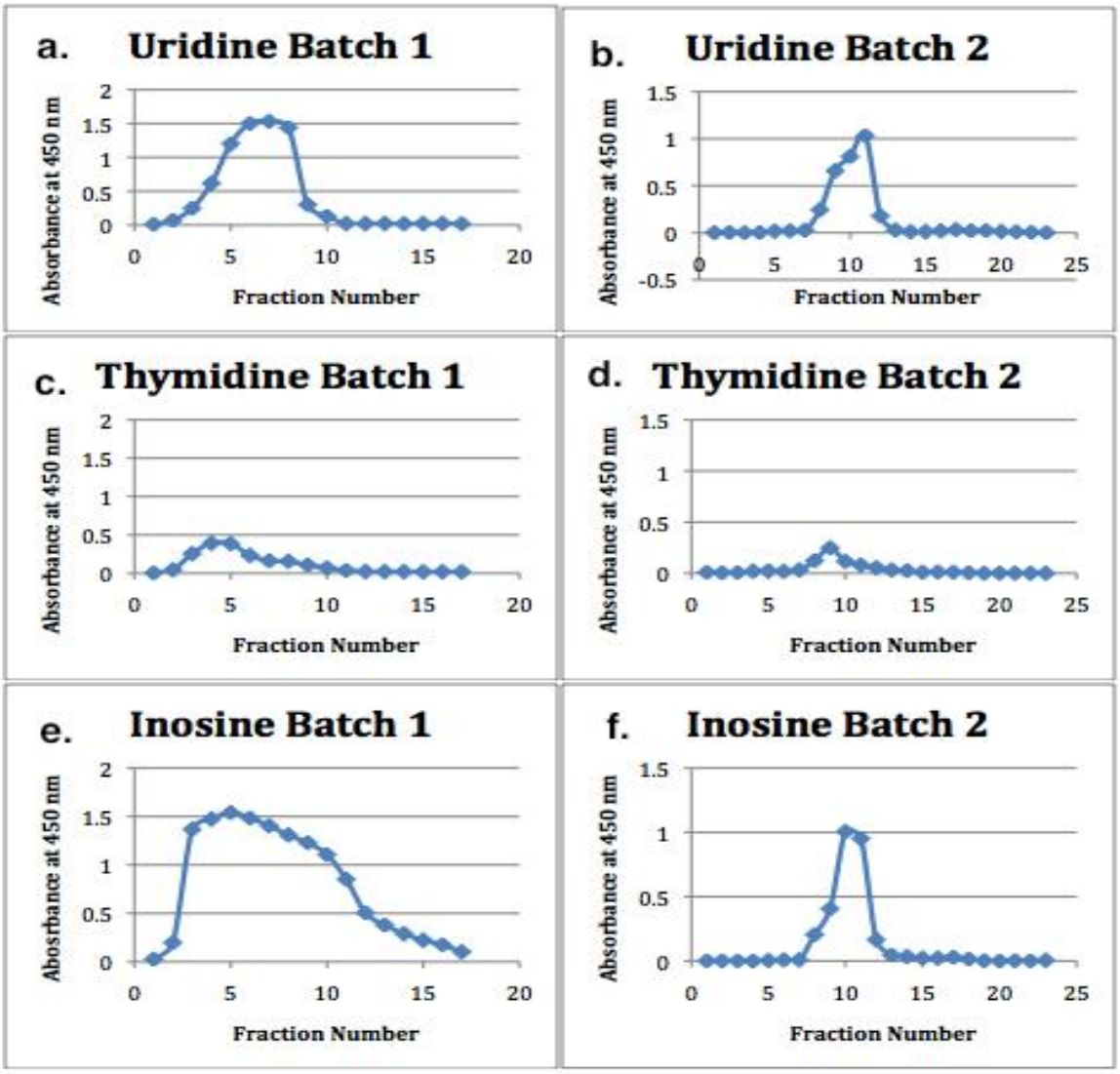


Figure 15. Activity elution profile for UPP, TPP, and IPP from Sephacryl S100 size exclusion column (a) One mL of each fraction is added to separate test tubes, each containing a uridine reaction mixture. The chromatogram indicates the strongest UPP content in Fractions 5-8 (b) In the second batch, the strongest UPP content is in Fractions 9-11. (c) One mL of each fraction is added to separate test tubes, each containing a thymidine reaction mixture. The chromatogram indicates a miniscule amount of TPP content in Fractions 3-6. (d) In the second batch, a very small amount of TPP is detected in fraction 9. (e) One mL of each fraction is added separate test tubes, each containing an inosine reaction mixture. The chromatogram indicates a very strong IPP content in Fractions 2-12 (f) In the second batch, the strongest IPP content is in Fractions 10-11.

Fractions 5-8 (batch 1) and 9-11 (batch 2) demonstrated the highest amount of UPP and were therefore collected and pooled into a single sample. Of course, this sample still contained high levels of IPP because as the chromatograms show, the elution peak of IPP was broader than that of UPP, which made separation impossible at this stage. However, the level of activity against thymidine was much lower and does not necessarily indicate the presence of TPP, as UPP is expected to accept thymidine as a substrate to a limited degree (Table 2).

3.5 Purification by Bio-Rad CHT Hydroxyapatite column

To separate UPP from IPP, a hydroxyapaptite column was next used. Hydroxyapatite is a naturally occurring crystalline form of calcium phosphate and has the molecular formula $Ca_{10}(PO_4)_6(OH)_2$.[63] Typically, a phosphate buffer is used to equilibrate the column. Three primary modes of interaction between the solid matrix and the proteins are responsible for separation. Immobilized phosphate groups create negative charges along the column, to which positively charged proteins bind. They may then be eluted into separate fractions by increasing the phosphate concentration or by addition of salt. Alternatively, addition of Ca^{2+} or Mg^{2+} will neutralize the phosphate charge and cause the proteins to elute as well. Negatively charged proteins may also become retained in the column as a result of either electrostatic repulsion or by complex formation between the carboxylic acid groups of the protein and the calcium sites on the column.[63] As with the size exclusion column, the sample was split into two batches and, in each case, a linear gradient of sodium phosphate buffer ranging from 10 mM to 800 mM was used as the mobile phase.

Figure 16 shows the resulting reducing sugar assay chromatograms for the first and second batches (4 mL and 3 mL, respectively). Just as with the Mono Q ion exchange and the Sephacryl S100 size exclusion columns, the absorbance of ribose is measured at 450 nm for each fraction to determine enzyme activity for UPP (Fig. 16a and 16b), TPP (Fig. 16c and 16d) and IPP (Fig. 16e and 16f).

As the chromatograms show, a significant amount of UPP eluted through the hydroxyapatite column in the wash while running the first batch. This did not happen in the second batch, which may be attributed to the fact that a 4 mL sample was loaded onto the column during the first run while the second batch only contained a 3 mL sample size. The additional 1 mL may have been enough to result in a saturated column with excess protein being eluted in the wash. Also noteworthy is the fact that no IPP eluted in the wash. This is advantageous as it provided a means to separate the two proteins. Fractions 2-9 were collected into a single sample and assayed by HPLC for activity against uridine. Figure 17 shows six resulting chromatograms which track the conversion of uridine to uracil over the course of 973 minutes. The high amount of nucleoside being converted to base indicates that UPP is indeed present in this sample. The same sample was then assayed by HPLC again for activity against thymidine and Inosine to rule out the presence of TPP and IPP. Figure 18 shows the results.

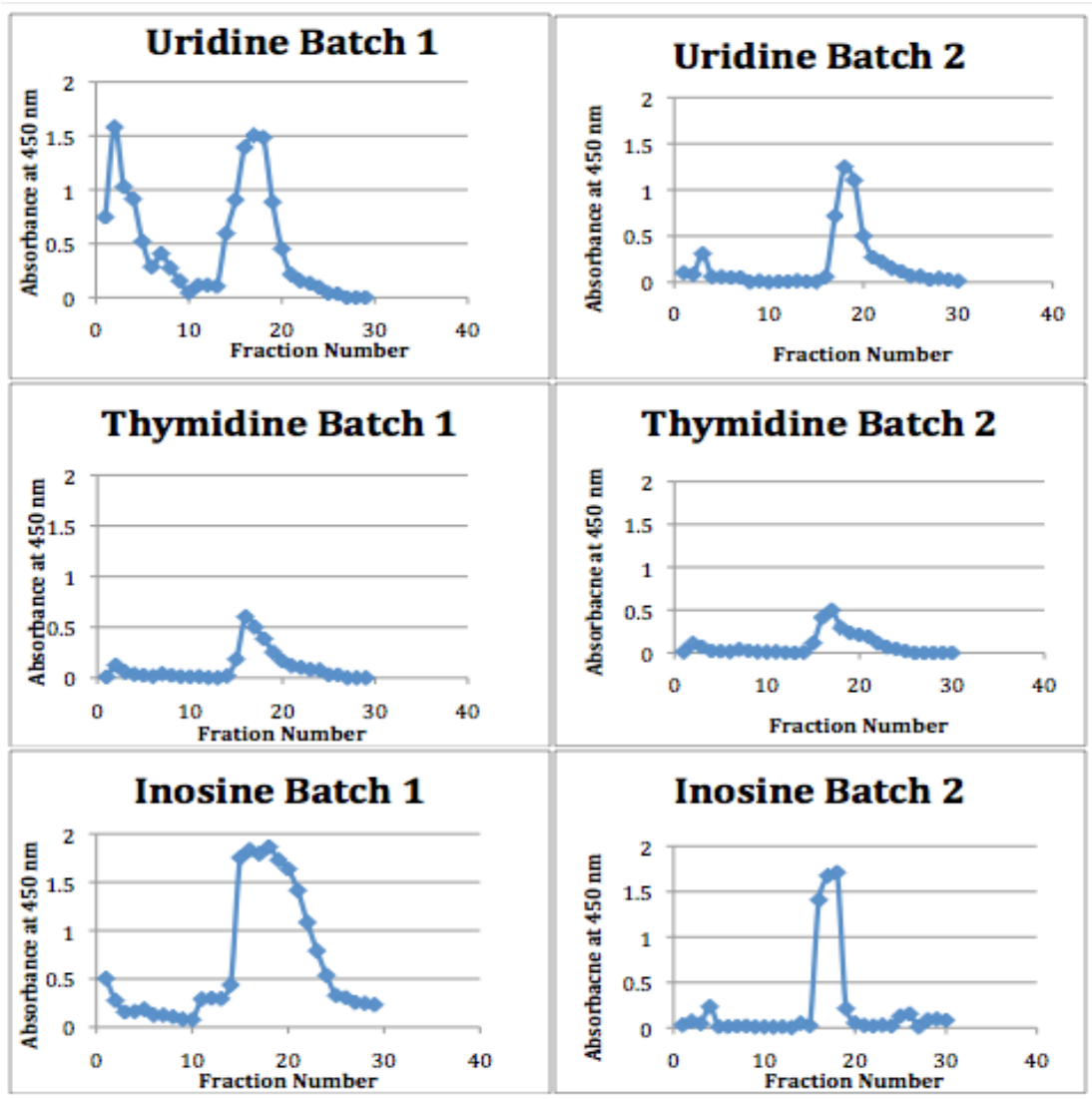


Figure 16. Activity elution profile for UPP, TPP, and IPP from Bio-Rad CHT hydroxyapatite column (a) One mL of each fraction is added separate test tubes, each containing a uridine reaction mixture. The chromatogram indicates UPP content in Fractions 1-9 and 14-20. (b) In the second batch, hardly any UPP came out in the wash, and a strong presence of UPP was seen in Fractions 17-20. (c) One mL of each fraction is added to separate test tubes, each containing a thymidine reaction mixture. The chromatogram indicates a very small amount of TPP content in Fractions 16-17 . (d) In the second batch, a very small amount of TPP is again detected in Fractions 16-17. (e) One mL of each fraction is added separate test tubes, each containing an inosine reaction mixture. The chromatogram indicates IPP content in Fractions 15-22 (b) In the second batch, the strongest IPP content is in Fractions 16-18.

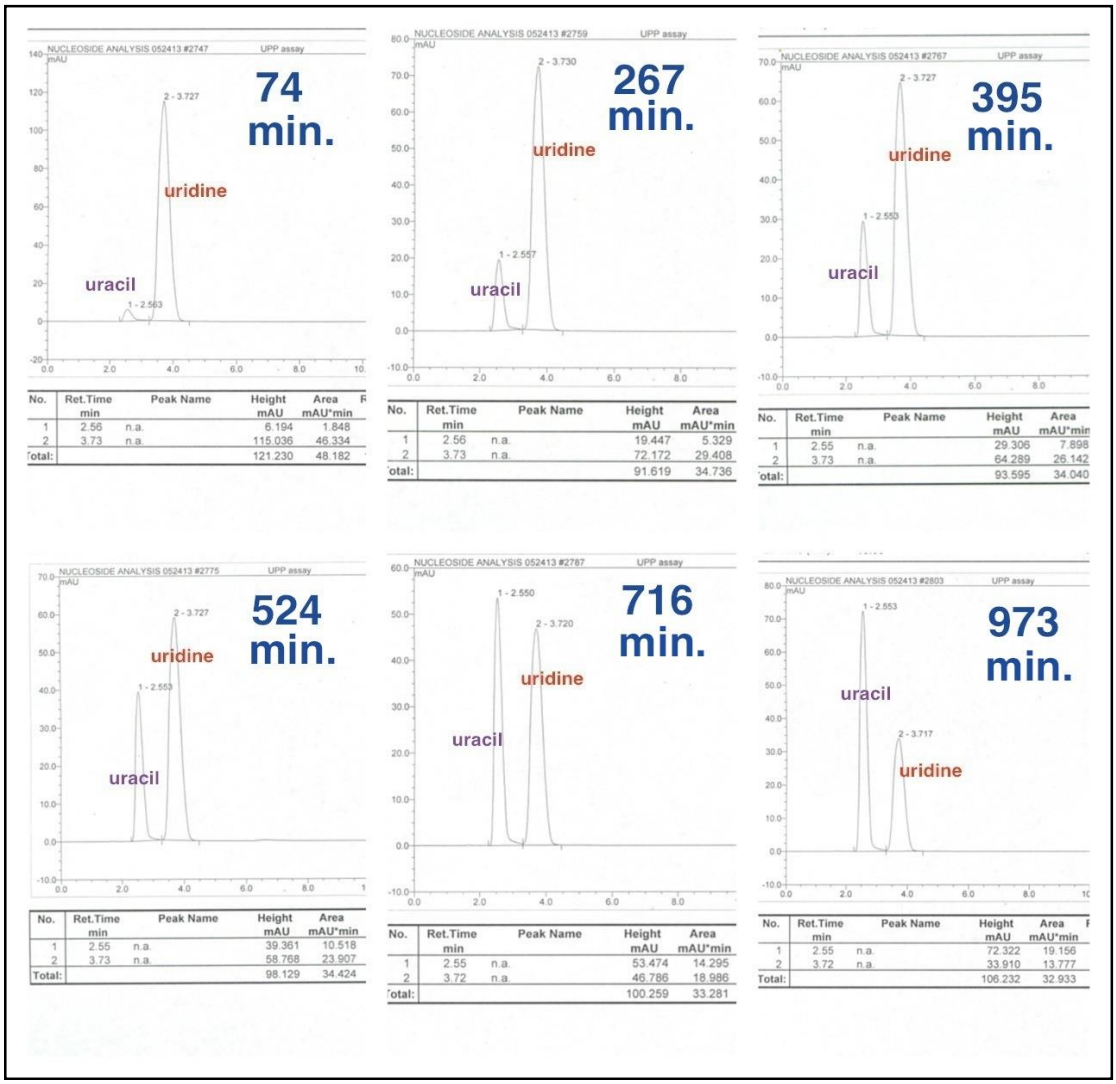
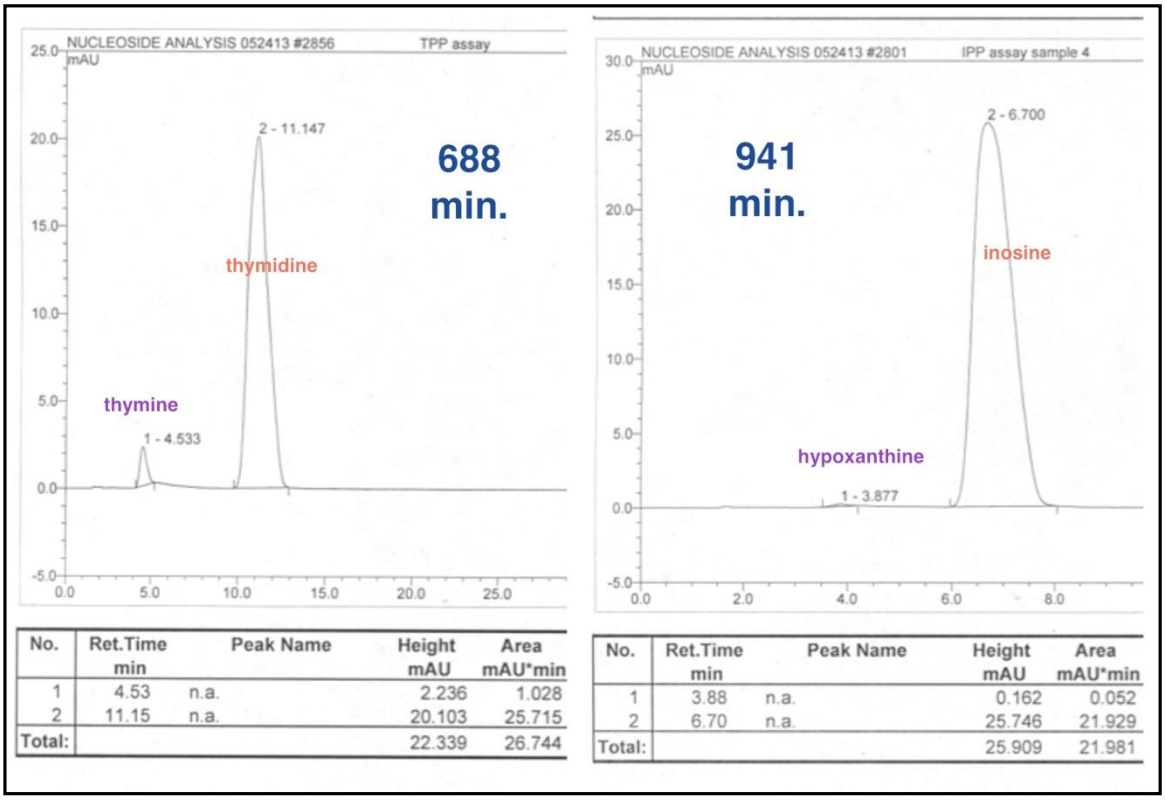


Figure 17. Activity assay by HPLC for UPP The conversion of uridine to uracil indicates the presence of UPP. The large numbers indicate the reaction time.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min
1	4.53	n.a.	2.236	1.028
2	11.15	n.a.	20.103	25.715
Total:			22.339	26.744

No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min
1	3.88	n.a.	0.162	0.052
2	6.70	n.a.	25.746	21.929
Total:			25.909	21.981

Figure 18. Activity assay by HPLC for TPP and IPP A small amount of thymidine has been converted to thymine over the course of 688 minutes. This supports the conclusion that UPP is present without TPP, as UPP typically accepts thymidine as a substrate to a smaller degree than it will accept uridine. If TPP were present, more activity would be expected against thymidine. No activity is shown for the conversion of inosine to hypoxanthine. This supports the conclusion that IPP is not present in this sample.

As shown in Figure 18, only a small amount of thymidine was converted to thymine after 688 minutes. UPP from other mammalian sources has been shown to accept thymidine as a poor substrate. It is reasonable to attribute the percentage of thymidine converted into thymine shown in Figure 18 as being due to UPP, as it is within bounds of what may be expected from a UPP enzyme isolated from mammalian liver tissue. For example, in a study conducted by el Kouni and coworkers, hepatic UPP isolated from murine liver showed the substrate activity for thymidine at 5% of the substrate activity for uridine.[17] This is in contrast to human

hepatic UPP, which did not show any tendency to cleave thymidine. Additionally, it was shown that TPP's ability to catalyze the conversion of thymidine to thymine is 13 times more efficient than UPP's ability to catalyze the conversion of uridine to uracil in human liver (1.3 times more efficient in mouse liver).[17] It is therefore logical that if TPP were present in this sample, at least as much activity would be shown against thymidine as is shown for uridine. Taking all of this into consideration, it is reasonable to conclude that UPP is the only enzyme present in this sample, although the appearance of a single band after gel electrophoresis would be needed for confirmation.

3.6 Determination of substrate specificity for UPP

The substrate specificity of UPP was determined for uridine, thymidine and inosine by comparing the velocities at which UPP catalyzes the conversion of each nucleoside to its respective base plus α ribose-1-phosphate. In order to determine the velocity, a 2 mM nucleoside reaction mixture was made for uridine, thymidine, and inosine. UPP (100 μL) was added to each reaction mixture. Each sample was then assayed by HPLC for at least 10 hours with 40-60 minutes between samples, resulting in 10-15 chromatograms per sample. The disappearance of nucleotide was then plotted against reaction time to get the velocity at which UPP catalyzed the reaction. These velocities are recorded and compared below in Table 4.

Table 4. Comparison of velocities for the conversion of substrates: uridine, thymidine, and inosine into respective bases

Substrate	Velocity (uM/min)	Relative Velocity
Uridine	0.3248	5
Thymidine	0.0161	1
Inosine	0	0

As Table 4 shows, bovine liver UPP coverts uridine to uracil at a rate 5 times greater of the rate at which converts thymidine to thymine. UPP showed no observable velocity with inosine as the substrate. This is consistent with previous observations of UPP, as there is no recorded instance of UPP accepting inosine as a substrate. UPP typically accepts thymidine to a lesser degree than uridine, although the ratios vary.

3.7 UPP purification table

After each step of the purification process, several parameters of the resulting sample were measured and recorded including: total volume (mL), total protein (mg), and the velocity at which the enzyme cleaves nucleoside (μM/min). The Bio-Rad assay procedure was used to determine the total protein in the sample, and the velocity of the enzyme is equal to the slope of the best-fit line between data points relating time vs. the disappearance of nucleoside. From these data, other parameters were able to be determined, including total activity (nmol/min), specific activity (nmol/min*mg), purification fold, and % recovery. For each step, the velocity was

divided by the amount of enzyme in the HPLC sample vial (100μL), then multiplied by the total volume to provide a value for the total activity of the sample. This value was then divided by the total protein in the sample to provide the specific activity, which is equal to the activity per mg. Dividing the specific activity for each step of the purification process by the initial specific activity (specific activity after the first step) provided the purification fold. As expected, this value increased each time, indicating that each step of the purification process was successful in removing unwanted proteins from the sample. Dividing the total activity after each step by the initial value for total activity (total activity after first step) and multiplying by 100 provided the % recovery values. As expected, this value decreased with each step of the purification process, as loss of a portion of the target protein is inevitable while removing unwanted proteins. Table 5 summarizes the results of the purification process.

As the data in Table 5 show, the hydroxyapatite column was the most critical step in this purification scheme. Although this step resulted in an 81.5% loss in protein from the previous step, it also resulted in a 16-fold purification, which far exceeds the purification achieved by the other columns. Conversly, the size exclusion S100 column only resulted in a purification fold of 1.41, while 56.44% of UPP was lost. Overall, the results presented indicate that the procedure used is an effective method for purifying UPP from bovine liver, however; successful final analysis by gel electrophoresis is needed to confirm that the end result is indeed a pure sample of UPP.

Table 5. Uridine phosphorylase purification table

Purification step	Total Volume (mL)	Total Protein (mg)	Velocity (μ/min)	Specific Activity ((nmoles/min)/mg)	Total Activity (nmoles/min)	Purification fold	% recovery
30% supernatant	100.00	1176.00	0.49	0.41	485.40	1.00	100.00
60% pellet	40.00	523.00	0.76	0.58	304.72	1.41	62.78
Mono Q	10.00	138.00	1.97	1.43	197.00	3.46	40.59
S-100	10.00	34.70	0.70	2.01	69.81	4.87	22.91
Hydroxyapatite	6.00	0.74	0.32	26.51	19.49	64.24	4.01

CHAPTER IV

CONCLUSION

In summation, uridine phosphorylase (UPP) appears to have been isolated from bovine liver, however SDS-PAGE would be needed as a final step to confirm this conclusion. UPP was found to coelute with two other proteins, thymidine phosphorylase (TPP) and inosine phosphorylase (IPP). TPP was found to have a very similar elution profile as UPP, although activity decreased to a very small amount after the Sephacryl S100 size exclusion column. IPP had a very broad elution profile that, in most cases, encompassed the fractions in which UPP eluted. The greatest challenge encountered was isolating UPP from IPP, and separation of the two was not achieved until the last step in the purification process. To begin the isolation process, the initial extract (cytosol of bovine liver cells) was precipitated with ammonium sulfate at 30% and 60% saturation. The 60% ammonium sulfate pellet was then resuspended and dialyzed against 10 mM sodium phosphate. The dialyzed sample was loaded onto a HiLoad Sepharose Fast Flow Mono Q column (16/10). The resulting fractions were assayed for protein at 280 nm and for activity at 450 nm by reducing sugar assay using uridine, thymidine, and inosine as substrates. The fractions that showed the most activity against uridine were pooled into a single sample then concentrated. The concentrated sample was then loaded onto a Sephacryl S100 (26/60) size exclusion FPLC column. Again, the fractions were assayed for protein at 280 nm and activity at 450 nm, using uridine, thymidine and inosine as substrates. The fractions showing the highest amount of activity for UPP were pooled,

concentrated, and dialyzed against 10 mM sodium phosphate buffer pH 7.2. The dialyzed sample was loaded onto a Hydroxyapatite column, and the eluted fractions assayed for protein at 280 nm and activity at 450 nm. Once again, uridine, thymidine, and inosine were used as substrates. The majority of UPP coeluted with IPP; however, an activity peak was present for UPP in the wash, which was not seen with IPP. These fractions were pooled and concentrated. The concentrated sample of UPP was assayed using HPLC for activity against uridine, thymidine, and inosine. The velocities at which UPP converts uridine, thymidine, and inosine into uracil, thymine, and hypoxanthine, respectively, were compared to determine the substrate specificity of UPP. The relative velocities were 5:1:0, respectively. UPP showed no observable activity with inosine as a substrate. The activity with uridine as a substrate was 5 times greater than the activity with thymidine as a substrate. The continuation of this project will be to assay the final purification process using gel electrophoresis to confirm that UPP has been completely isolated to a pure enzyme. The next step would be to determine the transition state of the enzyme. Enzymes bind most tightly to their substrates during the transition state; therefore, analogs that resemble the transition state would be expected to bind more tightly than the natural substrate and would serve as potent competitive inhibitors[9]. The application of this acquired knowledge would significantly aid in the improvement of existing inhibitors, and the development of new, more potent and more specific inhibitors.

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	(TK), thymidine synthase (TS), deoxyuridine triphosphatase (DUT), methylene tetrahydrofolate reductase (MTHFR). Metabolites: deoxyfluorocytidine riboside (5′-dFCR), deoxyfluorocytidine monophosphate (5′-dFCMP), deoxyfluorouridine monophosphate (5-FdUMP), deoxyfluorouracil (5′-dFUR), fluorouracil (5-FU), fluorouridine (5-FUridine), fluorouracil monophosphate (5-FUMP), fluorouracil di, tri-phosphate (5-FUDP, 5-FUTP), deoxyfluorouracil di, tri-phosphate (5-FdUDP, 5-FdUTP), deoxyuridine mono, tri –phosphate (dUMP, dUTP), deoxycytidine mono, tri-phosphate (dTMP, dTTP), 5,10-methylenetetrahydrofolate (5,10-MTHF), 5-methyltetrahydrofolate (5-MeTHF), dihydrofolate (DHF), dihydrofluorouracil (DHFU), beta-fluoroureido propionic acid (β-FUPA).
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