Nucleotide Metabolism

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OUTLINE:

1. Introduction
   a. General biological and clinical relevance
   b. Nomenclature
2. *De novo* biosynthesis: Purine and Pyrimidine
   a. Commonalities and differences between purine and pyrimidine synthesis
   b. Pyrimidine biosynthesis pathway
      -Clinical asides: DMARDs and hereditary orotic aciduria
   c. Regulation of pyrimidine biosynthesis
   d. Purine biosynthesis pathway
      -Clinical aside: Folate
   e. Regulation of purine biosynthesis
   f. Balancing nucleotide synthesis
3. Deoxyribonucleotide biosynthesis
   a. Ribonucleotide reductase function and regulation
   b. Thymidine synthesis
      -Clinical aside: 5-flurouracil/ DHFR inhibitors
4. Breakdown of nucleotides
   a. Breakdown of thymine
   b. Breakdown of purines
   c. Nucleotide breakdown clinical asides:
      i. ADA-SCID
      ii. Gout/ Allopurinol
5. Salvage pathway nucleotide synthesis
   a. Purine and pyrimidine salvage pathway enzymes
   b. Genetic defects in salvage pathway enzymes

LEARNING OBJECTIVES:

After studying this unit, you should be able to:

1. Recognize cell types that upregulate *de novo* synthetic pathways versus salvage pathways.
2. Compare and contrast basic cellular requirements for purine and pyrimidine *de novo* biosynthesis.
3. Name purine and pyrimidine nucleotides.
4. Identify the mammalian regulatory step in pyrimidine synthesis.
5. Explain the unique evolutionary mechanisms of the CAD enzyme.
6. Apply knowledge of the DMARDs discussed (mechanism of action, activity and function) to clinical scenarios.

7. Identify the cause of hereditary orotic aciduria.
8. Understand the mechanism of action of treatment with uridine for hereditary orotic aciduria.
9. Identify major points of regulation for pyrimidine biosynthesis within the CAD enzyme (both positive and negative feedback).
10. Identify the committed step of purine synthesis.
11. Explain why folic acid supplementation is recommended in prenatal care.
12. Recognize points of positive and negative regulation within the purine biosynthesis pathway.
13. Describe why the cell has developed sophisticated mechanisms to balance nucleotide and deoxynucleotide production.
14. Identify the enzyme and co-factors required for deoxyribonucleotide generation from ribonucleotides.
15. Generally, describe the regulatory mechanism used by ribonucleotide reductase.
16. Identify folate analogs that target nucleotide biosynthesis and explain their therapeutic uses.
17. Rationalize the use of 5-fluorouracil as a chemotherapeutic.
18. Recognize the primary end products of nucleotide breakdown.
19. Identify potential clinical consequences of and treatments used for excess nucleotide breakdown.
20. Describe genetic defects in the savage pathways.
21. Catalog general uses for nucleoside/nucleotide analogs and other drugs targeting nucleotide metabolism.

READING REFERENCE:

1. Marks' Basic Medical Biochemistry: A Clinical Approach, 5th edition; Chapter 39

https://meded.lwwhealthlibrary.com/content.aspx?sectionid=249269218&bookid=2170

ACKNOWLEDGEMENTS:

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1. Introduction

1a. General biological and clinical relevance

Nucleotides

How does the cell use nucleotides??

- Activated precursors of nucleic acids (for DNA and RNA)
- Universal suppliers of energy (ATP, GTP)
- Nucleotide derivatives participate in biosynthetic processes (*e.g.* UDP-glucose)
- Essential components of signal-transduction pathways (*e.g.* cAMP, cGMP)
- Components of coenzymes (*e.g.* adenine in CoA and NAD)

Diet is an insufficient source of nucleotides so they must be synthesized.

Several enzymes of nucleotide metabolism are implicated in disease, and others are drug targets.

Drugs that target nucleotide metabolism (some examples, not a comprehensive list)

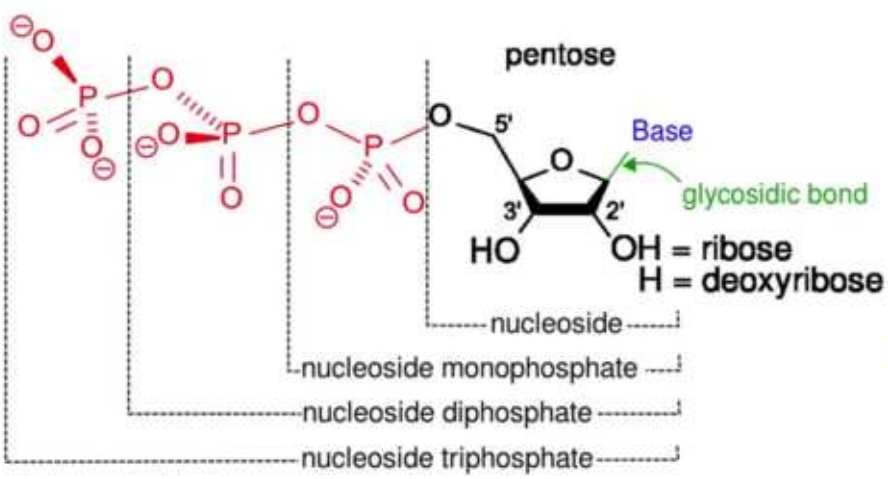
Drug	Pathway	Enzyme Target	Type	General Uses
Leflunomide	Pyrimidine synthesis	Dihydroorotate dehydrogenase	DMARD; prodrug, blocks access to enzyme active site	Autoimmune disease
Azathioprine (prodrug to 6-mercaptopurine)	Purine synthesis	GPAT	DMARD; prodrug, converted into thioguanine nucleotide	Autoimmune disease and transplant patients
6-mercaptopurine	Purine synthesis	GPAT	DMARD; prodrug, converted into thioguanine nucleotide	Acute lymphoblastic leukemia; off label for autoimmune disease
Mycophenolate	Purine synthesis	IMP Dehydrogenase	DMARD	Prevent graft rejection in transplant patients
Methotrexate	Folate metabolism, DNA synthesis	DHFR	DMARD/Anti-folate; Dihydrofolate mimetic	Chemotherapy for many cancer types and autoimmune disease
Trimethoprim	Folate metabolism, DNA synthesis	Bacterial DHFR	Anti-folate; Binds bacterial DHFR	Antibiotic
Pyrimethamine	Folate metabolism, DNA synthesis	Protozoa DHFR	Anti-folate; Binds protozoal DHFR	Antiparasitic
5-fluorouracil (Prodrug is capecitamine)	DNA synthesis	Thymidylate synthase	Nucleoside (uracil) analog	Chemotherapy
Allopurinol/ Febuxostat	Purine breakdown	Xanthine oxidase	Nucleoside/ Purine (hypoxanthine) analog	Gout, Nephrolithiasis, Tumor lysis syndrome
Remdesivir	Viral RNA replication and synthesis	Viral RNA polymerase	Nucleoside (adenosine) analog	Anti-viral; COVID-19
Acyclovir	Viral DNA replication	Viral DNA polymerase	Nucleotide (deoxyguanosine triphosphate) analog	Anti-viral; HSV, Shingles, Varicella
Ribavirin	Purine synthesis	IMP Dehydrogenase	Nucleoside analog	Antiviral; Hep C, RSV
Hydroxyurea	DNA synthesis	Ribonucleotide reductase	Antimetabolite	Chemotherapy, sickle cell anemia, psoriasis

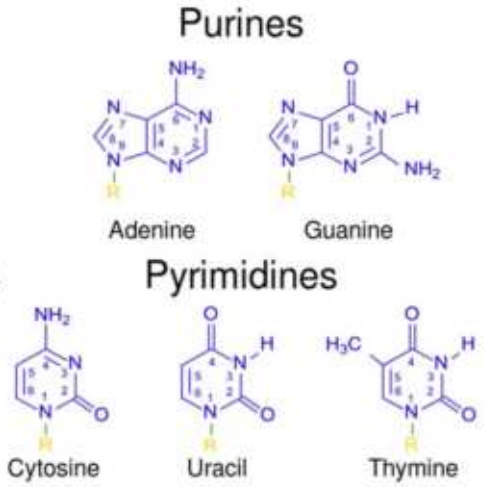
We will discuss the underlined drugs in more detail throughout this lecture, and they will be testable material for the block exam. All of the drugs in this table will likely be included in Step 1 testable content. I hope that as you proceed through the curriculum and encounter them in additional lectures, you will recognize their connections with nucleotide metabolism.

1b. Nomenclature

A reminder of the nomenclature

NTP NDP NMP



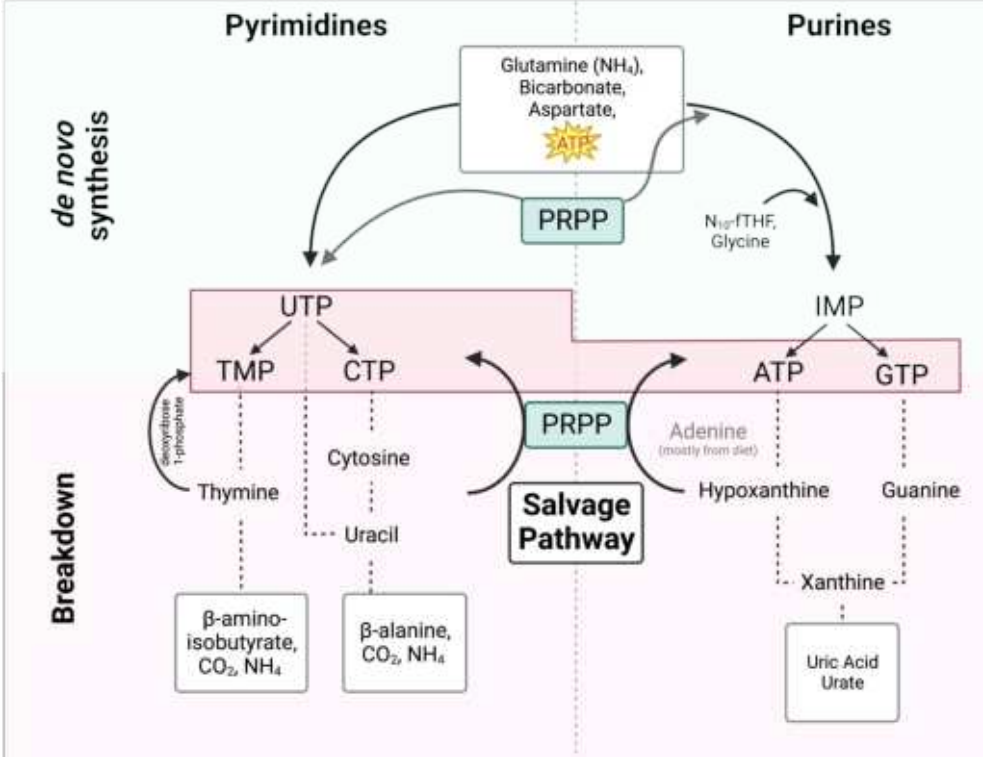


Biochemistry Free for All, http://oregonstate.edu/dept/biochem/ahem/123.html

TABLE 25.1 Nomenclature of bases, nucleosides, and nucleotides

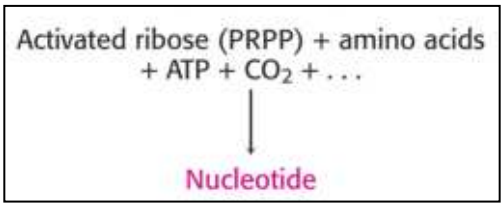
Base	Ribonucleoside	Ribonucleotide (5′-monophosphate)
RNA		
Adenine (A)	Adenosine	Adenylate (AMP)
Guanine (G)	Guanosine	Guanylate (GMP)
Uracil (U)	Uridine	Uridylate (UMP)
Cytosine (C)	Cytidine	Cytidylate (CMP)
DNA		
Base	Deoxyribonucleoside	Deoxyribonucleotide (5′-monophosphate)
Adenine (A)	Deoxyadenosine	Deoxyadenylate (dAMP)
Guanine (G)	Deoxyguanosine	Deoxyguanylate (dGMP)
Thymine (T)	Thymidine	Thymidylate (TMP)
Cytosine (C)	Deoxycytidine	Deoxycytidylate (dCMP)

Overview of Nucleotide Metabolism Pathways



Two pathways for nucleotide biosynthesis

1) De novo pathway: Building from scratch

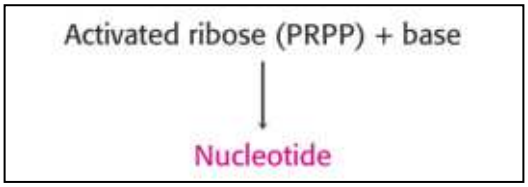


-*De novo* synthesis takes place when cells are rapidly growing and exhaust their existing supply of bases.
- -cancer cells
- -immune cells (B and T cells, for example)

-This pathway is targeted by drug companies for several important reasons:
- -Chemotherapies (e.g. 5-FU)
- -Therapies for over-active immune systems (e.g. Rhematoid Arthritis, lupus, Crohn's and other autoimmune diseases)

2) Salvage pathway: Adding ribose to an existing base

-Most other cell types primarily use the salvage pathway.

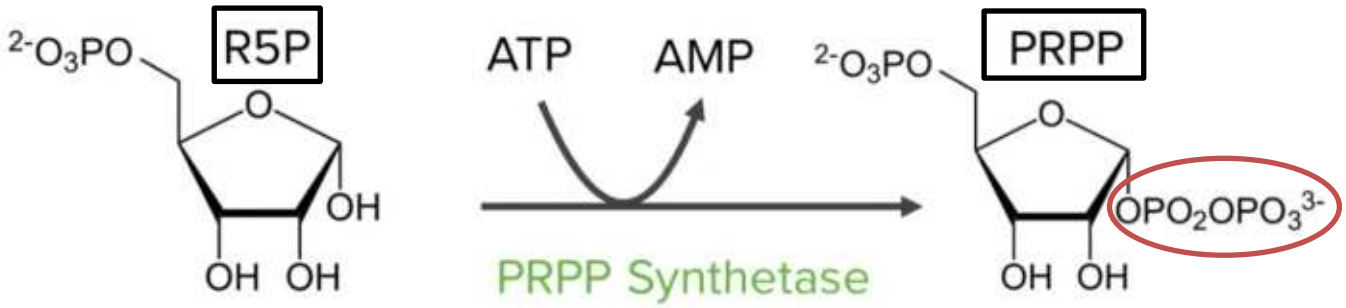


2. *De novo* biosynthesis: Purine and Pyrimidine

2a. Commonalities and differences between purine and pyrimidine synthesis

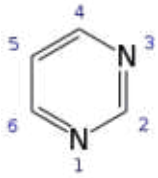
PRPP is a precursor for both the purine and pyrimidine pathways

- PRPP = 5-phosphoribosyl-1-pyrophosphate
- PRPP is an activated form of ribose; activated by ATP



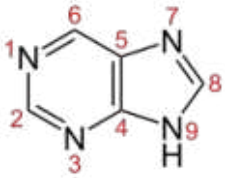
The pyrophosphate group added to ribose to make PRPP will become hydrolyzed in later reactions and provide the energy to drive those future reactions forward.

Pyrimidine vs purine biosynthesis



Pyrimidines

- Pyrimidine ring made then attached to ribose unit
- Less complicated
- Key precursors: bicarbonate, ammonia (glutamine) and aspartate
- Cytosine/ Uracil/ Thymine



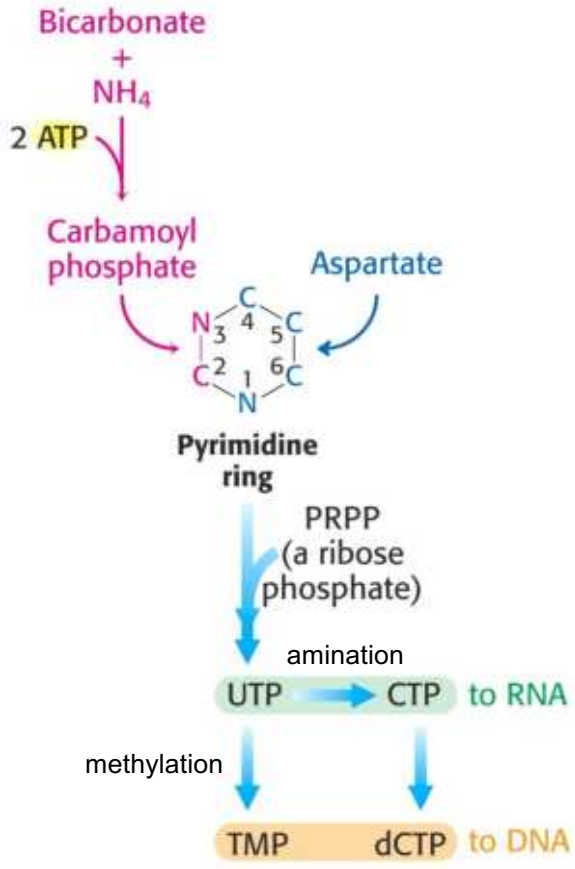
Purines

- Purine ring constructed on the ribose unit
- More complicated
- Key precursors: bicarbonate, ammonia (glutamine), aspartate, N^{10}-fTHF & glycine
- Adenine/ Guanine (Pure As Gold)

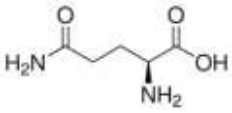
Both pathways require energy input (ATP)

2b. *De novo* pyrimidine biosynthesis pathway

Pyrimidine biosynthesis begins with bicarbonate, ammonia (generated by hydrolysis of the side chain of glutamine), and aspartate as precursors and through a series of reactions depicted on the next page makes UTP. UTP can be aminated to make CTP or methylated to make TMP and then TTP.



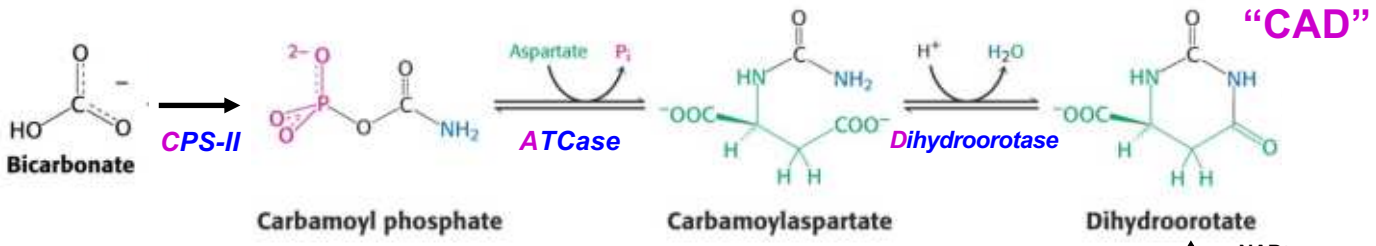
- Ring is synthesized first, then attached to ribose
- Precursors are bicarbonate, ammonia and aspartate
- Ammonia is typically produced from hydrolysis of the side chain of Glutamine (Gln)
- Requires ATP



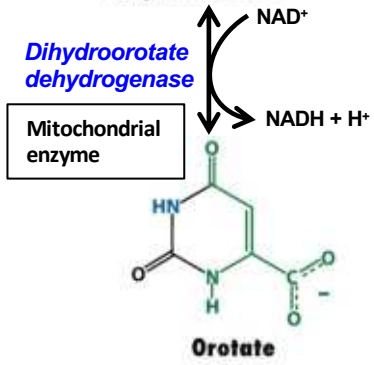
Glutamine

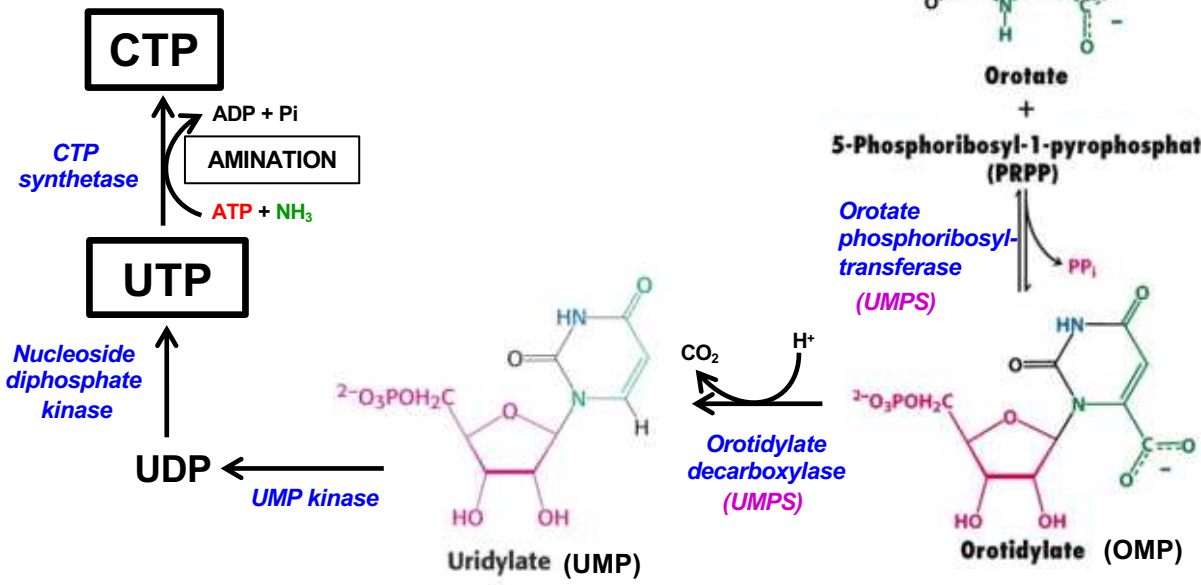
The ring structure above is color coded to indicate the atoms contributed by each precursor.

Overview of pyrimidine biosynthesis



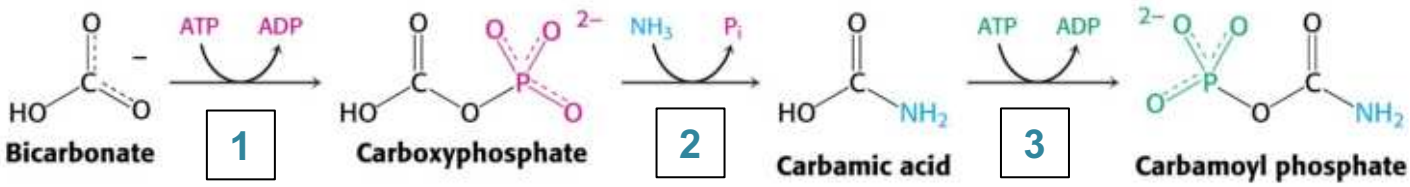
CPS-II = carbamoyl phosphate synthetase II
ATCase = aspartate transcarbamoylase





Reactions of pyrimidine biosynthesis: Synthesis of carbamoyl phosphate

- Multistep reaction
- All steps catalyzed by carbamoyl phosphate synthetase II (**CPS-II**)
- Uses 2 ATP (for phosphorylations)
- <u>Key regulated step in eukaryotes</u>



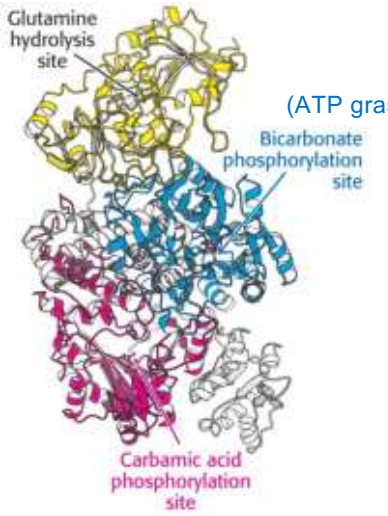
Phosphorylation of bicarbonate

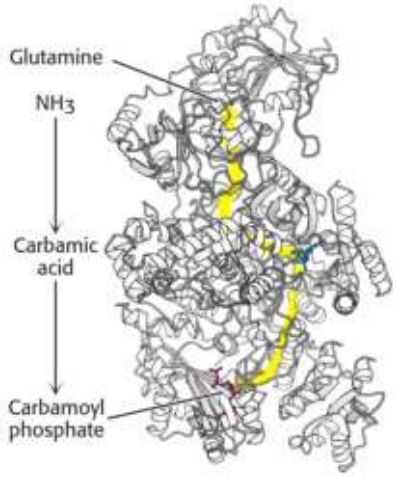
Ammonia replaces phosphate group

Second phosphorylation

Substrate channeling in CPS-II

Hydrolyzes Gln to provide ammonia to ATP grasp domain 1





- Prevents loss of intermediates via diffusion
- Labile intermediates are protected from hydrolysis (carboxyphosphate and carbamic acid)

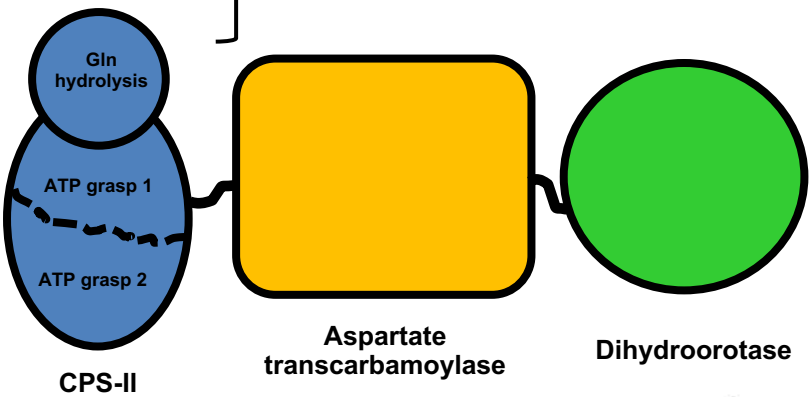
We saw this enzyme in Block 1 as an example of multi-domain enzyme efficiency mechanisms. Intermediates of the reactions catalyzed by CPS-II tunnel through the interior of the protein to their next active site, which increases the rate of the reactions by preventing loss of intermediates by diffusion and protects the intermediates from undergoing unwanted side reactions.

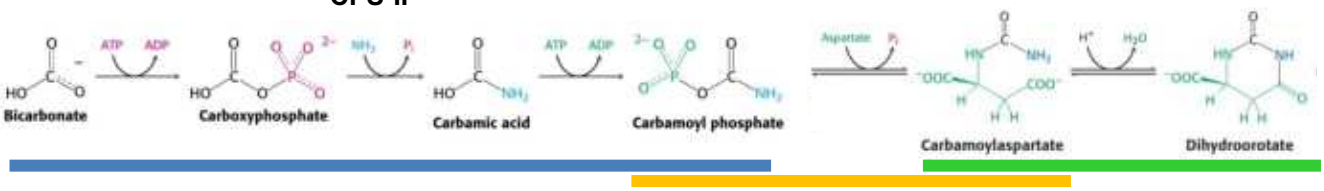
"CAD"

In mammals:

Carbamoyl phosphate synthetase II
Aspartate transcarbamoylase
Dihydroorotase

} present on one polypeptide chain – "CAD"





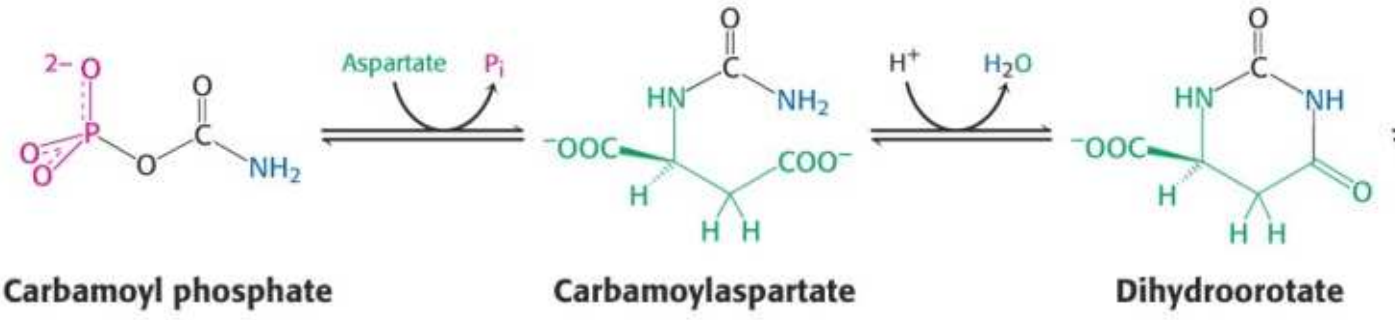
CPS-II has multiple domains that catalyze several reactions, but it is actually part of an even larger enzyme including aspartate transcarbamoylase and dihydroorotase domains as well (CAD). In lower organisms these are all separate enzymes but grouping them into one polypeptide chain has increased the efficiency of these reactions in mammals.

Carbamoyl phosphate to dihydroorotate

The two reactions below are the remaining reactions performed by the "CAD" enzyme in mammals. ATCase is the key regulated step in prokaryotes while CPS-II is the key regulated step in mammals. Impressively, in one enzyme ("CAD"), we have gone from bicarbonate to a closed 6-member ring structure (dihydroorotate).

Aspartate transcarbamoylase (ATCase)

Dihydroorotase



Amino group of Asp replaces phosphoryl

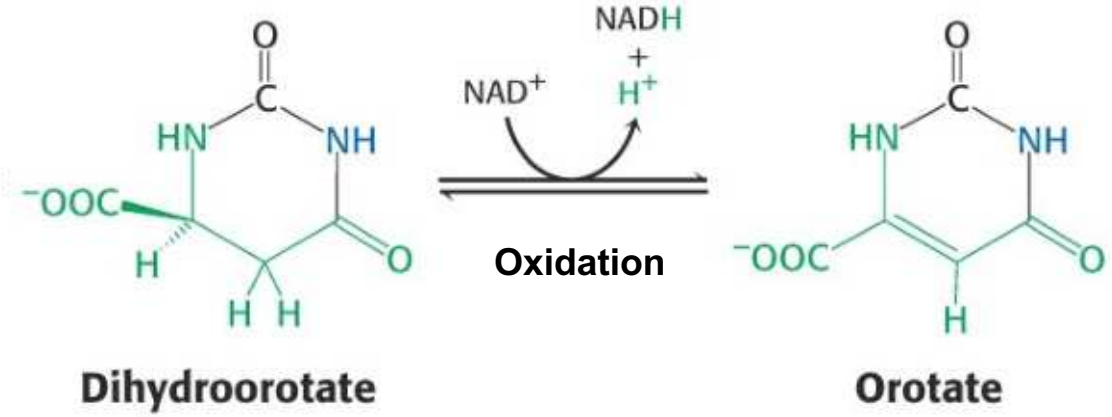
(regulated step in prokaryotes)

Dehydration/ Cyclization

Dihydroorotate to orotate

Next, dihydroorotate dehydrogenase oxidizes dihydroorotate to orotate, our precursor pyrimidine base generating one molecule of NADH in the process.

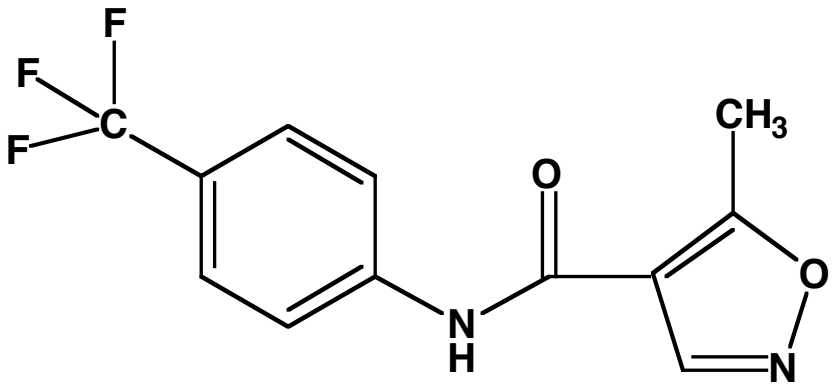
Dihydroorotate dehydrogenase



Oxidation

Orotate is the precursor pyrimidine base.

CLINICAL ASIDE: The DMARD leflunomide inhibits *de novo* synthesis of pyrimidines

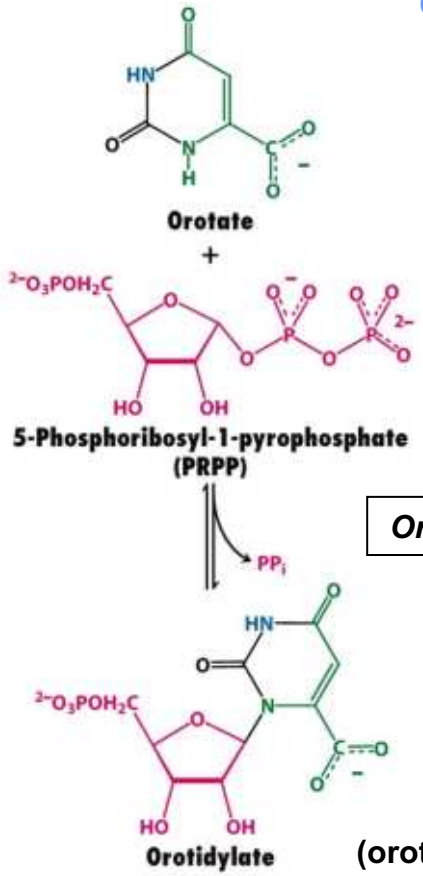


- Brand names: **Arabloc**, **Arava**, **Lunava**, **Repso**
- Used in the treatment of moderate to severe rheumatoid arthritis and psoriatic arthritis.
- Prodrug – is metabolized to teriflunomide
- Inhibits **dihydroorotate dehydrogenase** – binds to a tunnel leading to the active site
- Targets rapidly dividing cells, which require a lot of DNA
- Immunosuppressive because drug prevents expansion of activated lymphocytes

Disease-modifying antirheumatic drugs (DMARDS)

- Used to treat inflammatory diseases, *e.g.* rheumatoid arthritis, lupus, Crohn's and other autoimmune diseases
- There are many classes with various mechanisms of action
- Some target nucleotide metabolism:
  - Azathioprine – purine synthesis inhibitor
  - Leflunomide – pyrimidine synthesis inhibitor
  - Methotrexate – nucleotide metabolism inhibitor through dihydrofolate reductase inhibition (DNA synthesis)
- Generally, DMARDs work by inhibiting T cell expansion.

Orotate to orotidylate



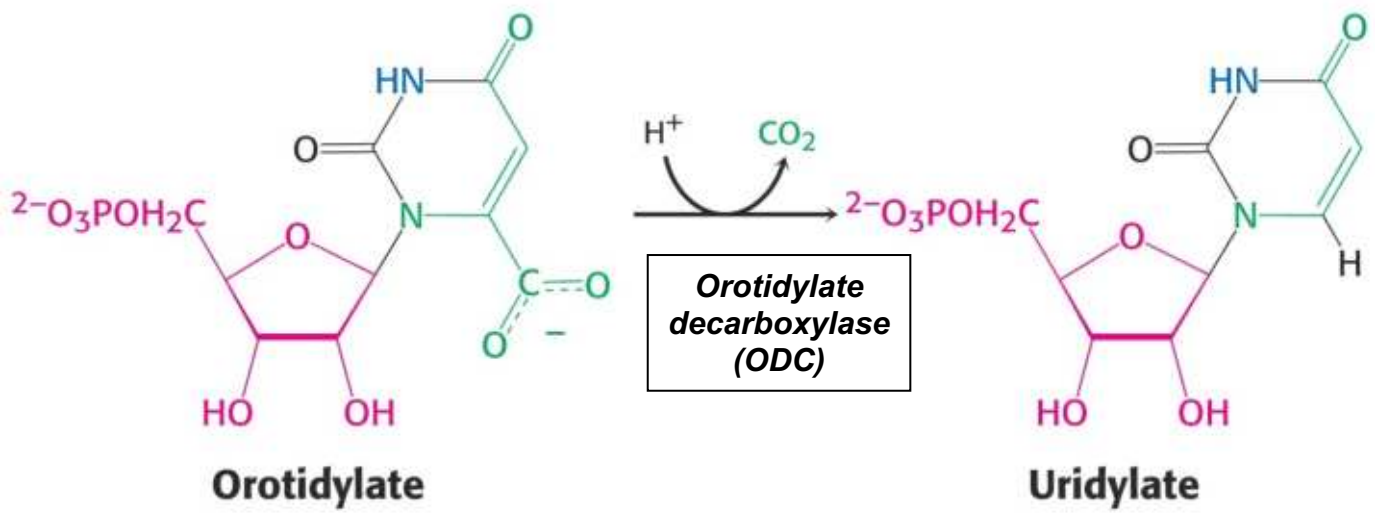
- Adding ribose 5-phosphate to the pyrimidine base using **PRPP**
- Reaction driven by hydrolysis of PP_i

Orotate phosphoribosyltransferase (OPRT)

If you see PRT in the enzyme name, that indicates that the PRPP molecule is being used in the reaction to attach ribose to the base. We will encounter several "PRT" enzymes.

(orotidine monophosphate – OMP)

Orotidylate to uridylate



Orotidylate decarboxylase (ODC)

(orotidine monophosphate – OMP)

(uridine monophosphate – UMP)

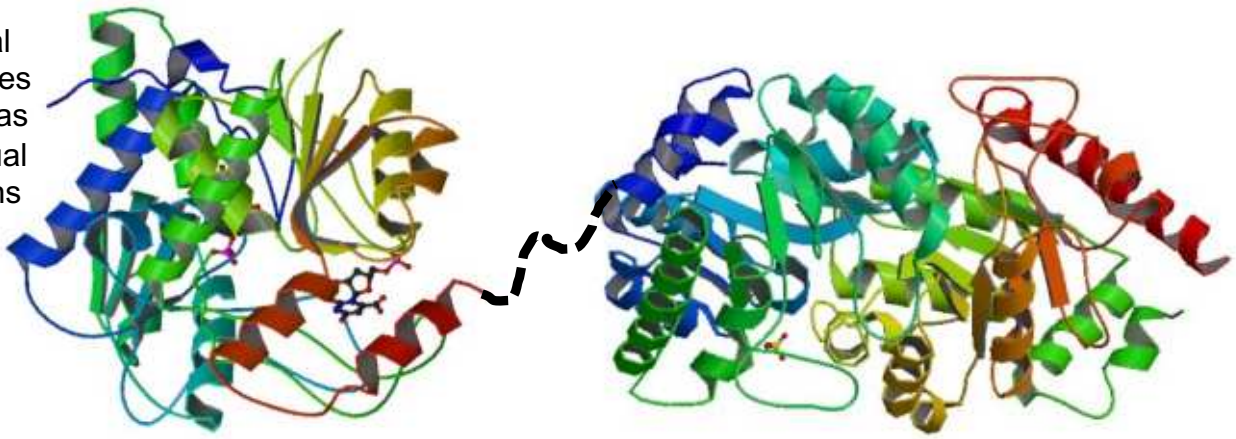
- Decarboxylation reaction
- Orotidylate decarboxylase is one of the most efficient enzymes known:
  - -rate enhancement is 10^{17}
  - -without enzyme there would be one decarboxylation every 78 million years!

Uridine monophosphate synthetase (UMPS)

In mammals, orotate phosphoribosyltransferase and orotidylate decarboxylase are part of the same polypeptide. Again, higher organisms have evolved to increase efficiency and the rates of reactions by encoding enzymes that are separate in lower organism within one gene and therefore one polypeptide chain.

OPRT and ODC =>bifunctional enzyme called UMPS

Crystal structures solved as individual domains



N-terminal domain - orotate phosphoribosyl transferase (OPRT)

C-terminal domain – orotidylate decarboxylase (ODC)

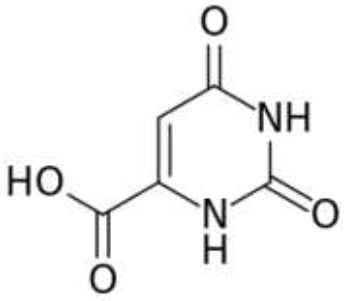
CLINICAL ASIDE: Hereditary orotic aciduria

Frequency: extremely rare, <20 cases worldwide

Symptoms: high levels of orotic acid in urine, megaloblastic anemia, severe mental and physical delays

Cause: autosomal recessive, defect in UMPS (orotate phosphoribosyltransferase or orotidylate decarboxylase)

Treatment: Uridine (=>UMP) to bypass metabolic block



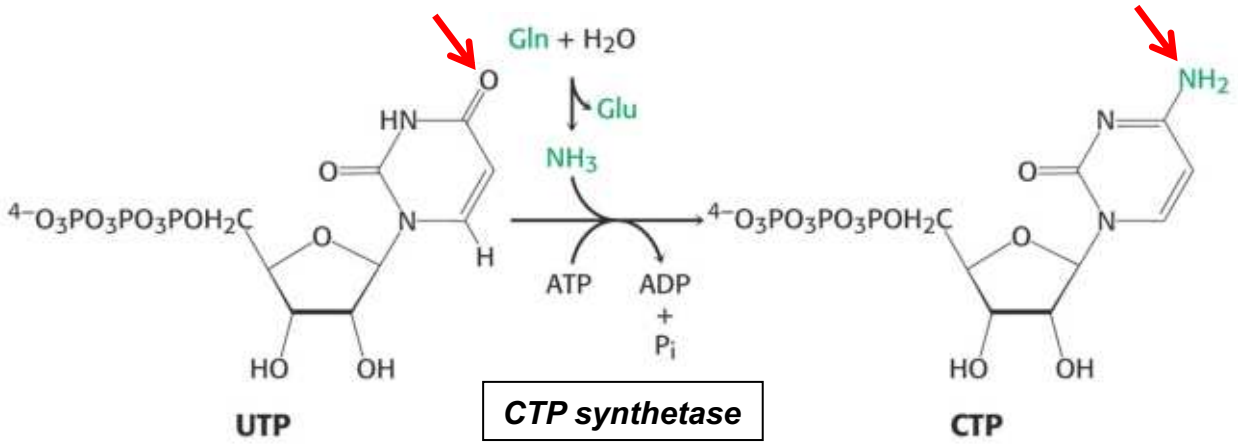
Orotic acid

Orotic aciduria can also arise due to blockage of the urea cycle (especially ornithine transcarbamylase deficiency)

-The two forms of the disease are separated by measuring blood ammonia levels and blood urea nitrogen (BUN).

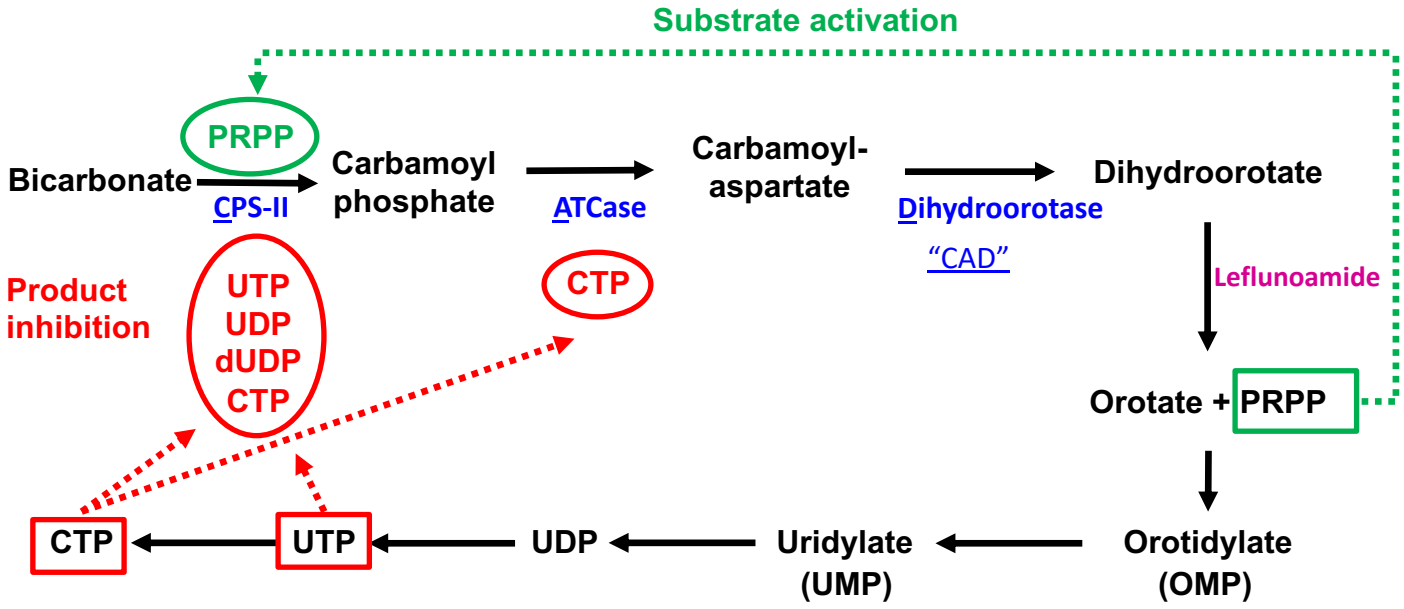
CTP is formed by amination of UTP

In the last step we discussed, UMP was synthesized. Two kinases phosphorylate UMP to first make UDP (UMP kinase) and then UTP (Nucleoside diphosphate kinase). UTP can then be aminated by CTP synthetase to form CTP (below) or methylated to form TTP as we will see later in the lecture. This reaction is very similar to the chemistry we saw performed by CPS-II.



- Replacement of the carbonyl by an amino group
- Enzyme mechanism is analogous to CPS-II
- Requires ATP
- Gln provides amino group again

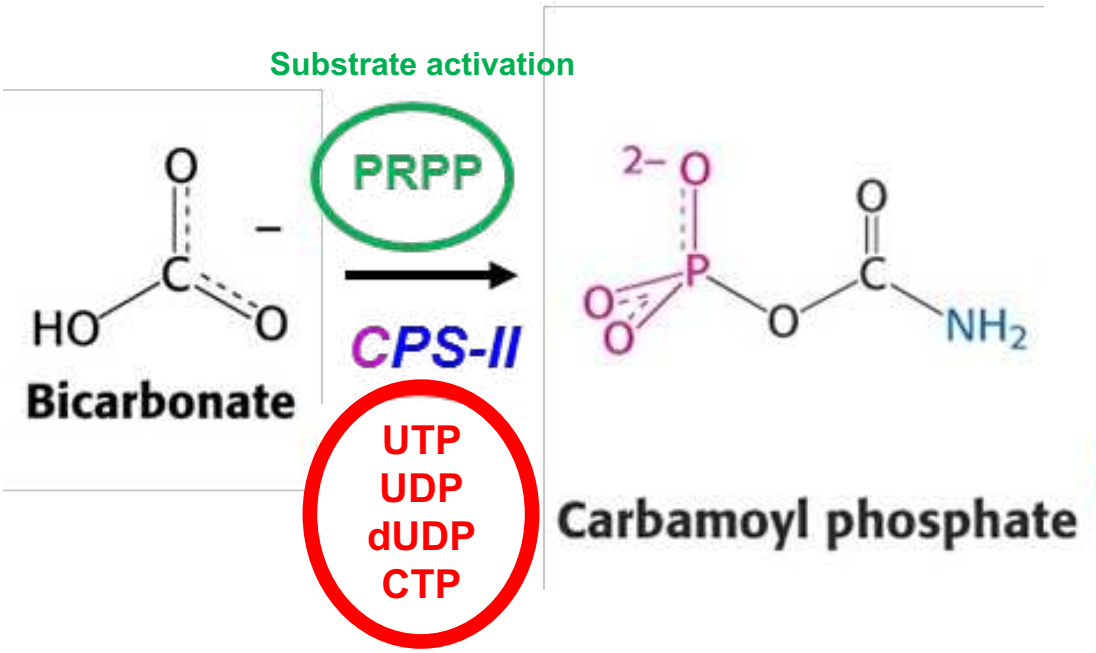
2c. Regulation of pyrimidine biosynthesis



***Defect in UMP synthetase causes hereditary orotic aciduria**

Pyrimidine biosynthesis utilizes typical substrate activation and product inhibition mechanisms. Nucleotide synthesis requires energy input. If the cell has CTP and UTP molecules that are not being used and are building up, then the cell does not want to waste energy making more. Therefore UTP, UDP, dUDP and CTP can all inhibit the CPS-II enzyme (our key regulated enzyme in mammals). CTP can inhibit ATCase (the key regulated enzyme in prokaryotes). On the other hand, a build up of the substrate PRPP will activate CPS-II to initiate pyrimidine synthesis.

CPS-II is the key regulatory step in eukaryotes

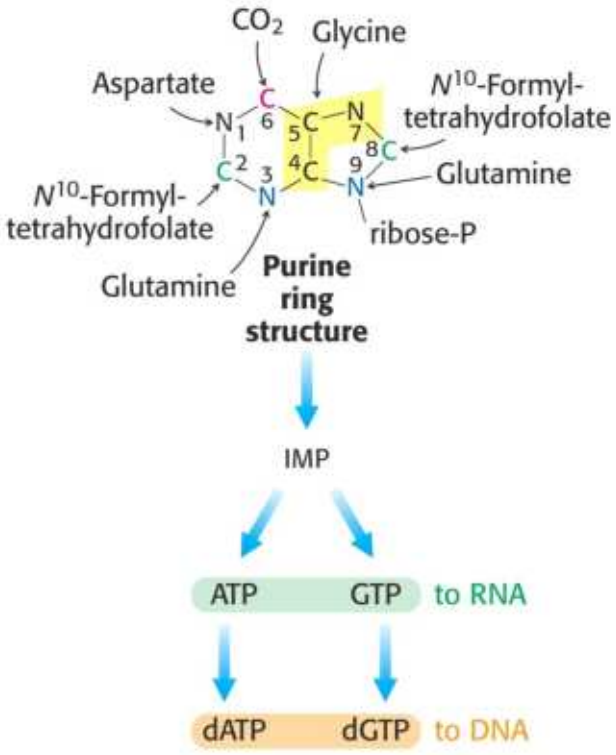


An additional layer of regulation of the CPS-II enzyme occurs during the cell cycle. A MAP kinase phosphorylates and sensitizes CPS-II to activation during S phase to accommodate the increased cellular demand for nucleotides during DNA replication. The enzyme is dephosphorylated at the end of S-phase.

Cell cycle control:

- In S-phase, DNA is synthesized and CPS-II becomes more sensitive to activation by PRPP and less sensitive to UTP inhibition
- The opposite occurs at the end of S-phase
- Mechanism is phosphorylation of CPS-II by a MAP kinase

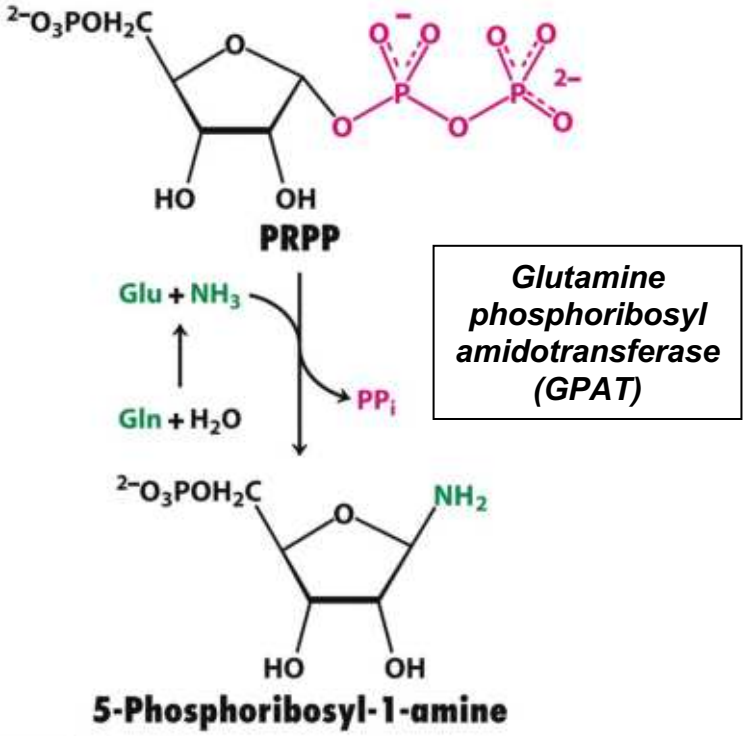
2d. *De novo* purine biosynthesis pathway



- Purine base is built while attached to the ribose
- More complicated
- N^{10}-fTHF & Gly involved

The purine double rings are larger and more complex than the pyrimidine single ring bases. Unlike pyrimidines which add ribose using PRPP after first building the precursor base, purines start with PRPP and build the base structure using ribose as a scaffold. The diagram on the left is again color coded to indicate the atomic contributions of the input molecules. Inosinate (IMP) is a purine precursor for both ATP and GTP.

Purine synthesis begins with PRPP

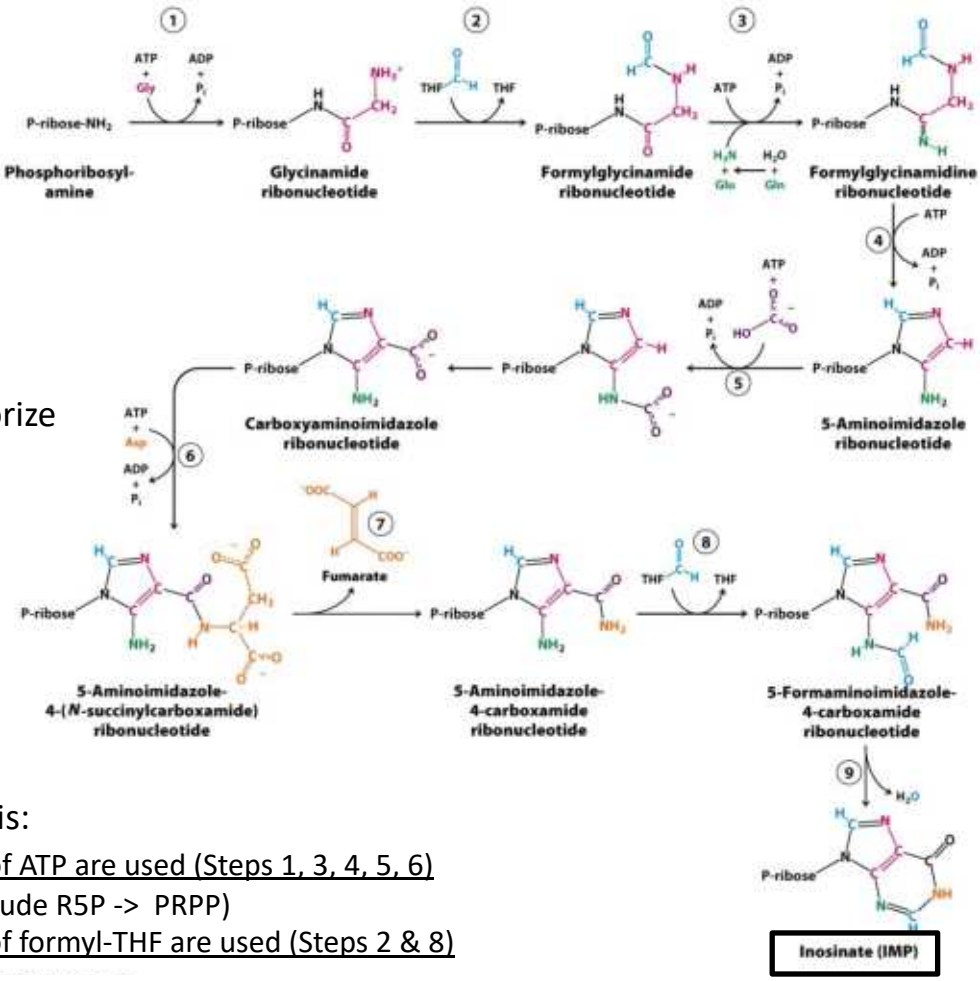


- Displacement of PP_i on PRPP by ammonia
- Committed step of pathway
- Highly regulated by feedback inhibition
- Two domains in enzyme:
  - -Gln hydrolysis domain
  - -amidotransferase domain
- Product unstable
- Target of azathioprine and 6-mercaptopurine (6-mp)

GPAT is the committed step of the purine biosynthesis pathway which uses the hydrolysis of the pyrophosphate group of PRPP to drive the amidotransferase reaction to generate 5-phosphoribosyl -1-amine. Similar to CPS-II, multiple domains are fused together to protect labile intermediates.

9 steps from phosphoribosyl-1-amine to IMP

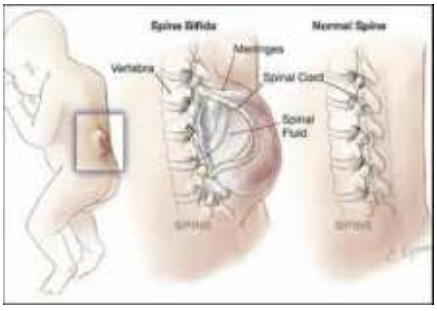
Don't memorize this…

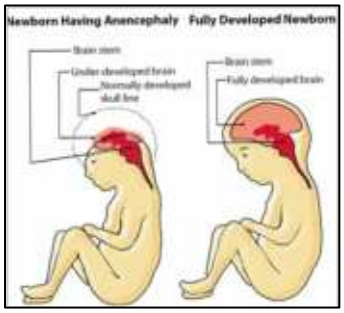


But know this:

-5 molecules of ATP are used (Steps 1, 3, 4, 5, 6)
(6 if you include R5P -> PRPP)
-2 molecules of formyl-THF are used (Steps 2 & 8)

Clinical aside: Folic acid in pregnancy





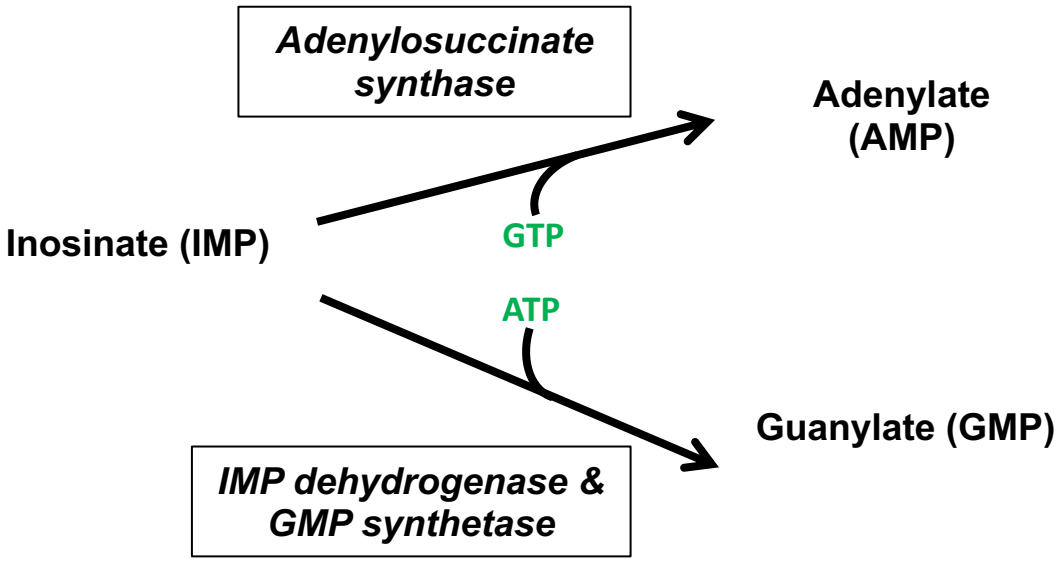
https://bscmalformations.weebly.com/neural-tube-defects.html

- Folate, or vitamin B9, is thought of as one of the 13 *essential* vitamins.
- *Folic acid* is a synthetic dietary supplement
- Neither folate nor folic acid is metabolically active. Both must be reduced to participate in cellular metabolism.

Demands for folate increase during pregnancy because it is also required for growth and development of the fetus.

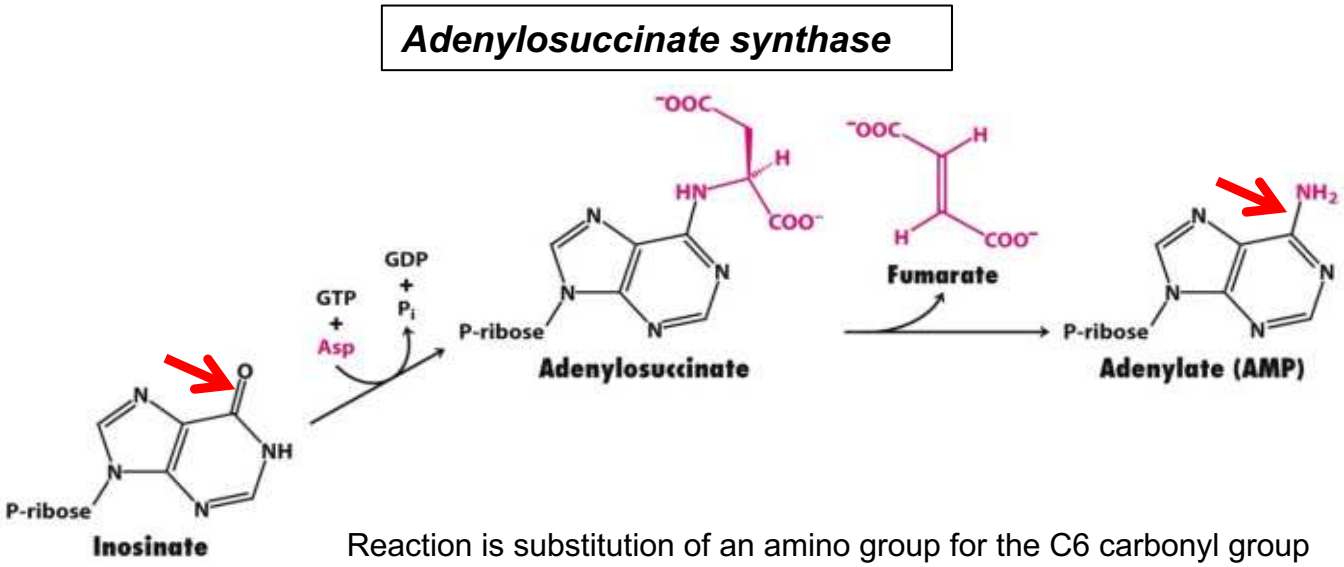
Folate deficiency has been associated with abnormalities in both mothers (anemia, peripheral neuropathy) and fetuses (congenital abnormalities).

Inosinate is a precursor for both adenylate and guanylate



Adenylosuccinate synthase, which forms AMP, requires GTP for the reaction. Alternatively, GMP synthetase, which is one of the enzymes that forms GMP, requires ATP for the reaction. These substrate requirements for ATP and GTP naturally balance AMP and GMP levels because increased synthesis of one will drive synthesis of the other. Simple and elegant mechanism to ensure equal abundance of nucleotides.

Inosinate (IMP) to adenylate (AMP)

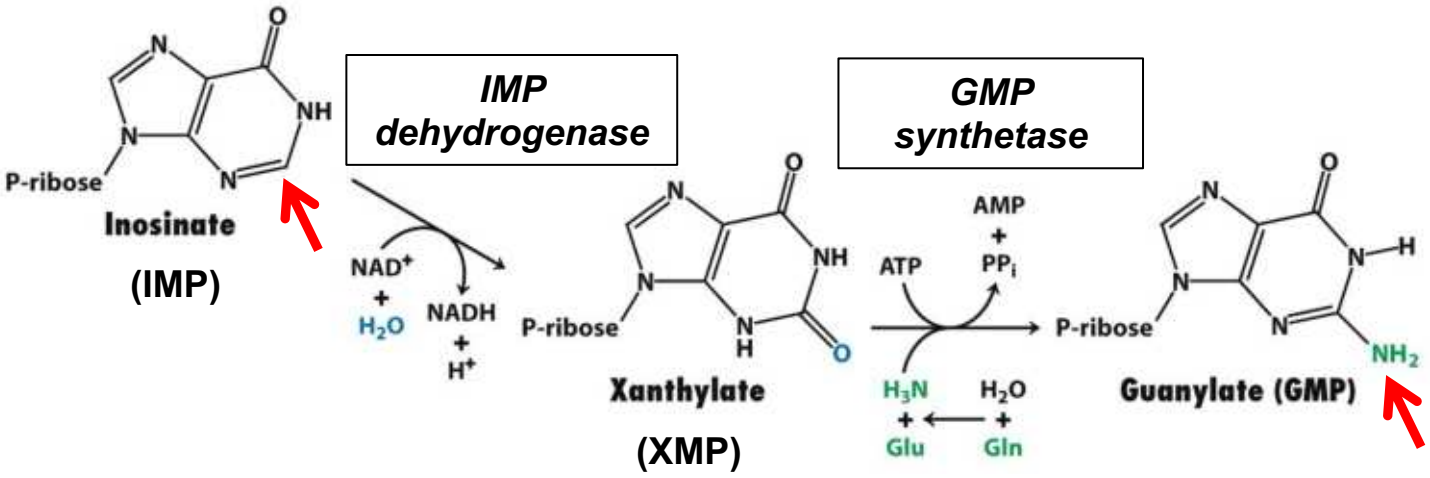


Reaction is substitution of an amino group for the C6 carbonyl group

- Amino group is produced by addition of Asp, followed by elimination of fumarate
- Reaction powered by GTP
- Enzyme related to G proteins

Adenylosuccinate synthase catalyzes a two-step reaction which replaces a carbonyl within inosinate with an amino group to form adenylate using GTP and an aspartate molecule in the processes.

Inosinate (IMP) to guanylate (GMP)



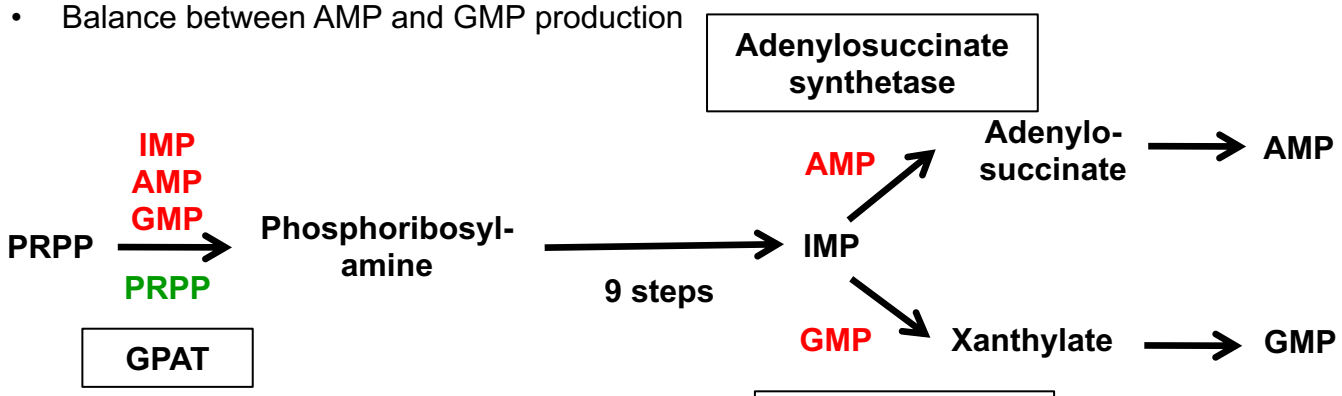
- Oxidation followed by incorporation of amino group at C2
- Ammonia produced by hydrolysis of Gln
- Xanthylate is activated by transfer of AMP to the carbonyl rather than a phosphoryl
- AMP displaced by ammonia

The conversion of inosinate to guanylate requires two enzymes. First, IMP dehydrogenase oxidizes IMP to a Xanthylate (XMP) intermediate adding a carbonyl. GMP synthetase replaces this carbonyl with an amino group derived from the hydrolysis of the side chain of glutamine using energy acquired from ATP. Interestingly, IMP dehydrogenase is the target of two clinically utilized therapies, the DMARD mycophenolate and the nucleoside analog ribavirin.

2e. Regulation of purine biosynthesis

Like pyrimidine regulation, purine biosynthesis is primarily regulated by substrate activation (PRPP) and product inhibition (IMP, AMP and GMP) of GPAT to control the overall activity of the pathway. AMP and GMP also feedback to inhibit adenylosuccinate synthetase and IMP dehydrogenase respectively.

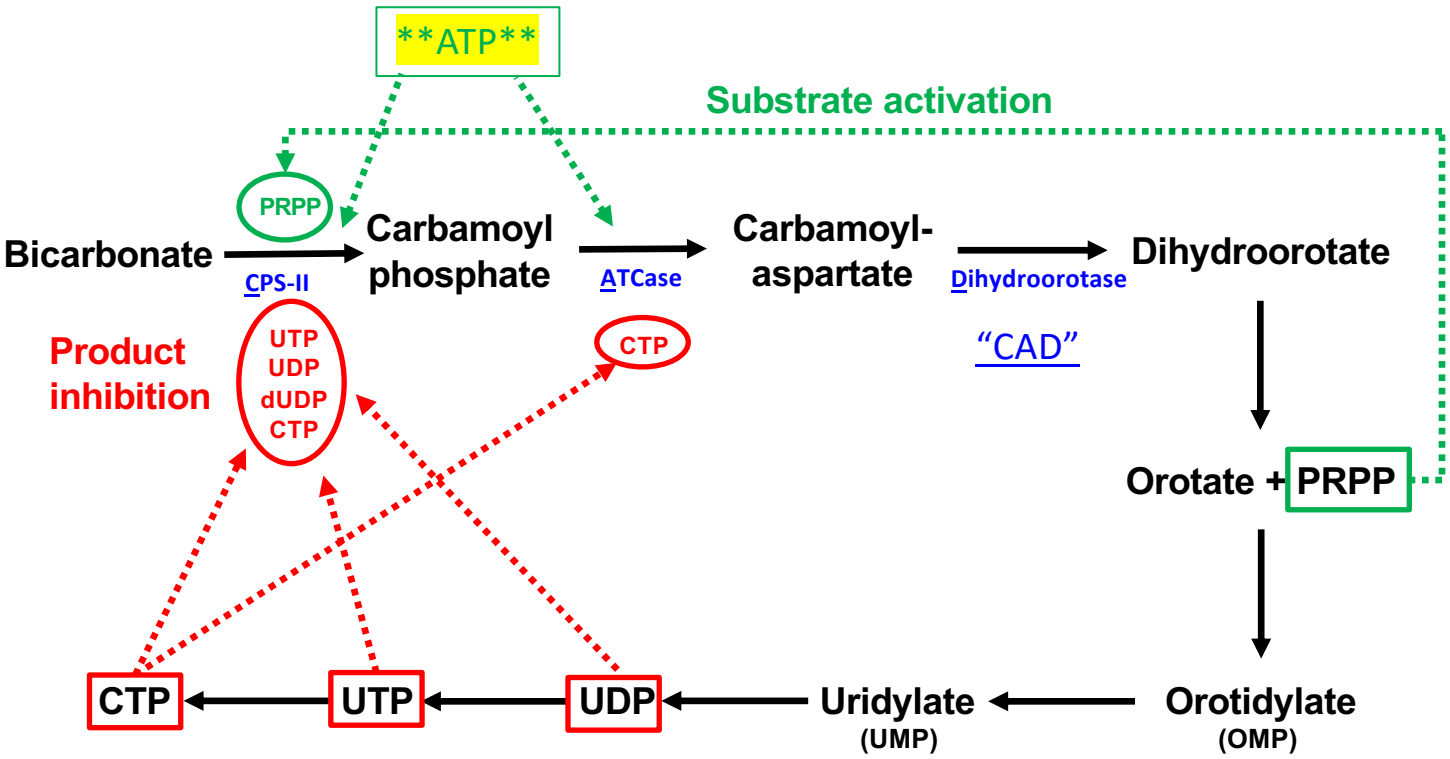
- Substrate activation and product feedback inhibition
- Balance between AMP and GMP production



•GPAT is the committed step in purine biosynthesis

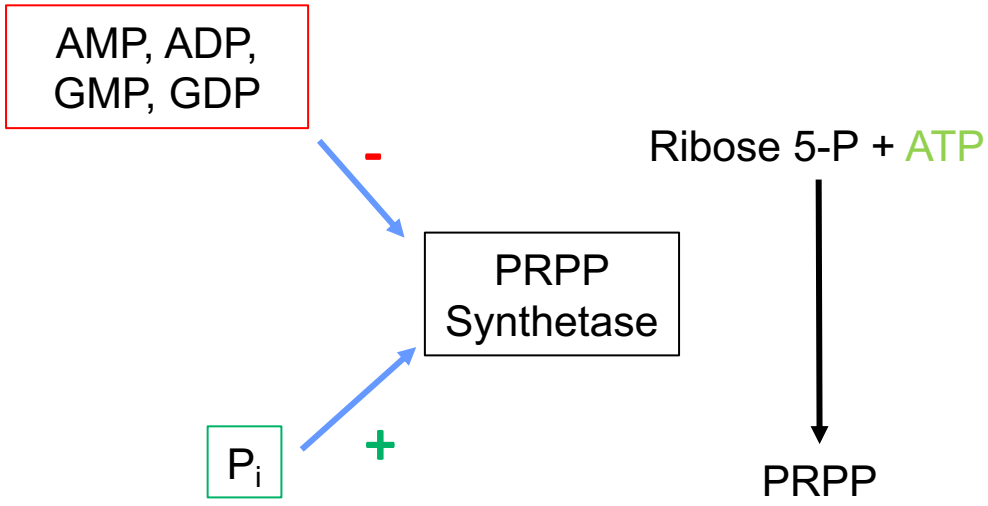
•Allosteric: Inhibition of GPAT by AMP and GMP is synergistic (either molecule alone only partially inhibits)

2f. Balancing purine and pyrimidine biosynthesis



Regulation of nucleotide synthesis is critical to balance the quantities of each nucleotide within the cell. For example, if one of the nucleotides is lacking in concentration, the cell risks mutations during replication as DNA polymerase may not be able to find the nucleotide when needed. We have already seen that ATP and GTP naturally balance each other in purine synthesis. Additionally, ATP activates the CPS-II and ATCase domains of CAD to maintain equal purine and pyrimidine nucleotide stores.

Regulation of PRPP synthetase:



Going back to the very beginning of the purine and pyrimidine biosynthesis pathways, we can see additional regulation of PRPP synthetase. ATP is required to provide the pyrophosphate group added to the ribose molecule, but high levels of AMP, ADP, GMP and GDP will inhibit the enzymes overall activity, indicating to the cell that it has sufficient nucleotide stores. On the other hand, high levels of inorganic phosphate will activate the enzyme.

3. Deoxyribonucleotide biosynthesis

The dNTP biosynthesis pathways are straightforward because we simply make deoxyribonucleic acids from ribonucleic acids. This simplicity is one piece of evidence for the RNA world hypothesis which alludes to a prebiotic era where early life forms relied on RNA for function and for storage of our genetic material.

RNA world hypothesis

Likely a ribozyme came first.
Then, the coding role was replaced by DNA (which is more stable than RNA) and the functional role was replaced by proteins (which are also more stable).

Evidence:

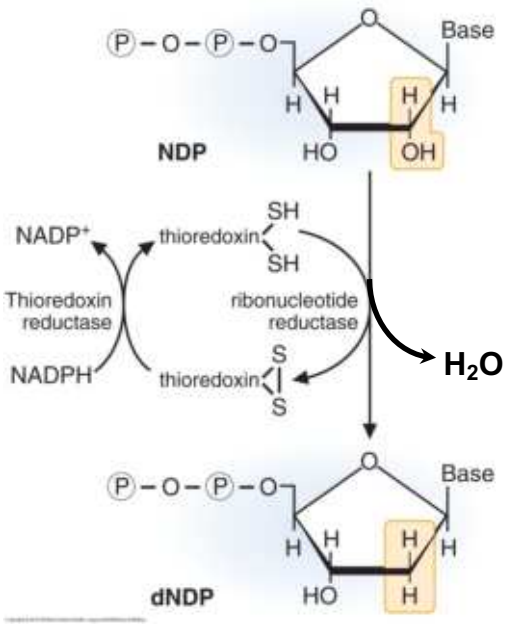
- Ribonucleotides are made first and then converted to deoxyribonucleotides by reduction of the ribose moiety.
- Thymine for DNA is made from uracil
- Deoxyribonucleotides are only used for DNA synthesis while ribonucleotides are used to make RNA and many cofactors (e.g. FAD, NAD, NADP, CoA, etc.)

https://www.synchrotron-soleil.fr/en/news/noncoding-rna-case-lc-ribozyme

3a. Ribonucleotide reductase function and regulation

Synthesis of deoxyribonucleotides by ribonucleotide reductase

One enzyme (ribonucleotide reductase) converts ribonucleotides to deoxyribonucleotides by removing the hydroxyl group at the 2' position of the ribose moiety as water. This reaction requires NADPH and the enzyme thioredoxin reductase to reduce the disulfide bond generate in the active site of ribonucleotide reductase during catalysis. NADPH is an important product of the pentose phosphate pathway that we will talk about extensively. This is one of the reactions within DNA synthesis that requires NADPH, while RNA synthesis does not require NADPH directly.



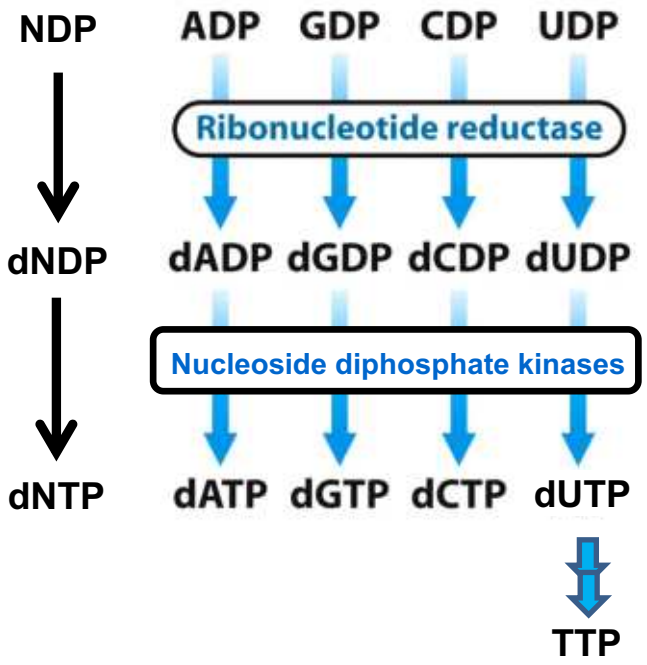
ribonucleotide

- Made by reduction of ribonucleotides
- Occurs at the dinucleotide level
- 2'-hydroxyl is reduced by thioredoxin => dNDP
- Reduced thioredoxin regenerated using NADPH (from pentose phosphate pathway)
- dNDPs made only for DNA synthesis

deoxyribonucleotide

Hydroxyurea, used as a chemotherapeutic and to treat sickle cell anemia and psoriasis, is an inhibitor of ribonucleotide reductase.

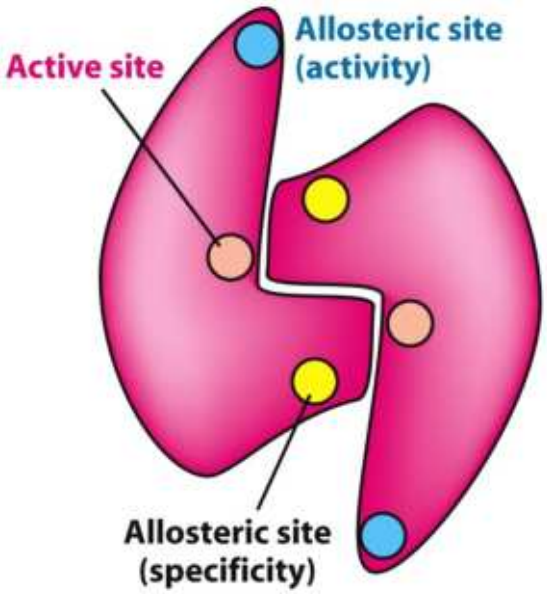
Ribonucleotide reductase reduces all four ribonucleotides

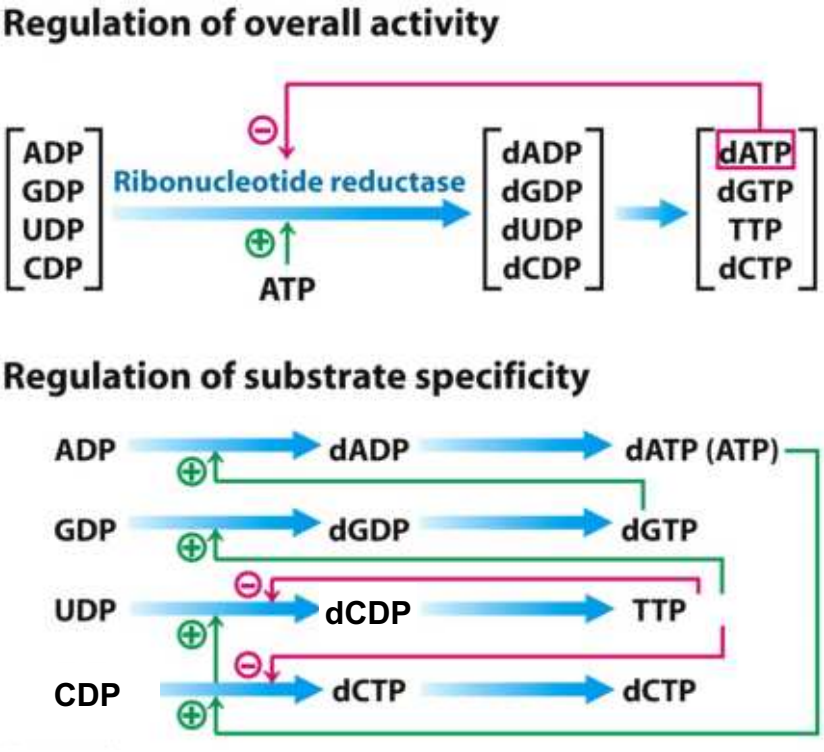


Importantly, deoxyribonucletide synthesis occurs at the diphosphate level by ribonucleotide reductase. Once converted to dNDPs, nucleoside diphosphate kinases must phosphorylate the nucleosides to generate the dNTPs required for DNA.

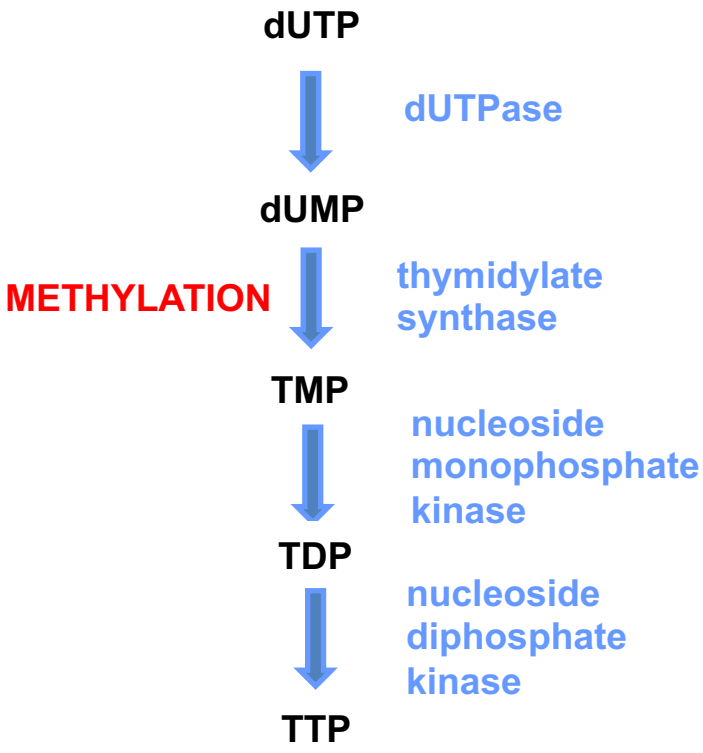
Regulation of deoxyribonucleotide synthesis by ribonucleotide reductase

Again, it is essential to balance deoxyribonucleotide stores in the nucleus as an imbalance could increase the chances of mutation during replication and repair. Ribonucleotide reductase plays a major role in maintaining this balance through the use of two allosteric sites within the enzyme. The activity allosteric site regulates the overall activity (increased dATP levels act through product inhibition and ATP activates (substrate activation)). The specificity allosteric site balances the levels of individual deoxynucleotides through the cyclical mechanism below. Build up of the concentration of one dNTP will activate synthesis of another dNTP. Follow the arrows below to see how each individual nucleotide will activate and inhibit the enzyme.





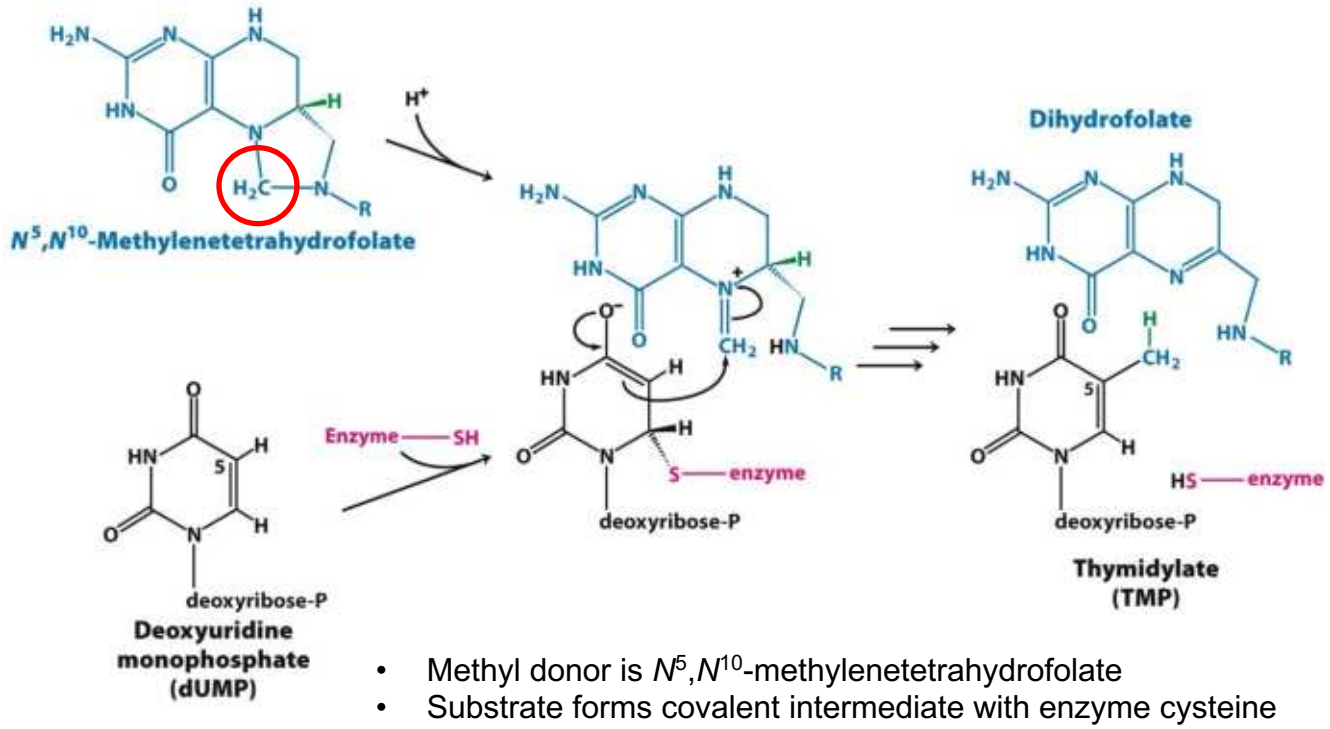
3b. Thymidine synthesis



The last synthesis cascade that we haven't discussed yet is thymidine. To make thymidine from dUTP, first the phosphates are removed by dUTPases to create dUMP. Methylation occurs at the monophosphate level by thymidylate synthase. TMP is then phosphorylated by nucleoside mono- and di –phosphate kinases to yield TTP.

Thymidylate synthase

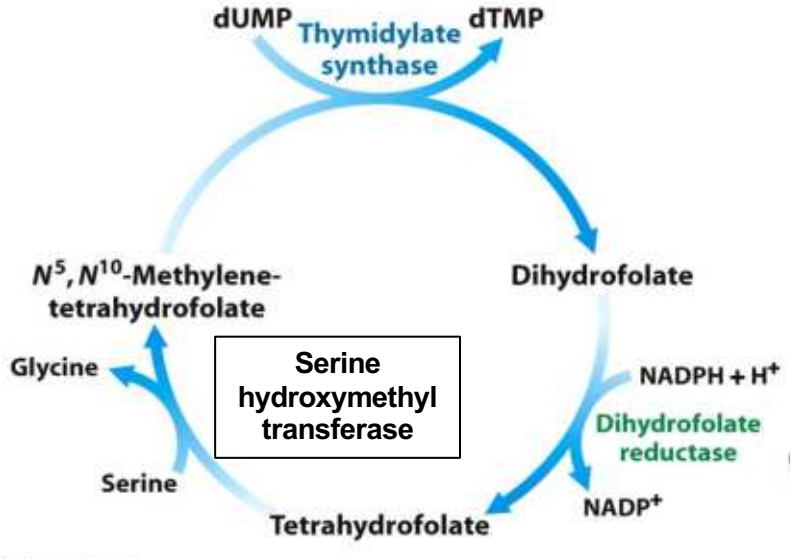
Below is the methylation step in more detail. Here, N^5, N^{10}-Methylenetetrahydrofolate is a folate derivative that is used as a single carbon donor to methylate the C5 position of dUMP to generate TMP. dUMP forms a covalent intermediate with the active site of the enzyme during catalysis before transfer of the methyl group and resolution.



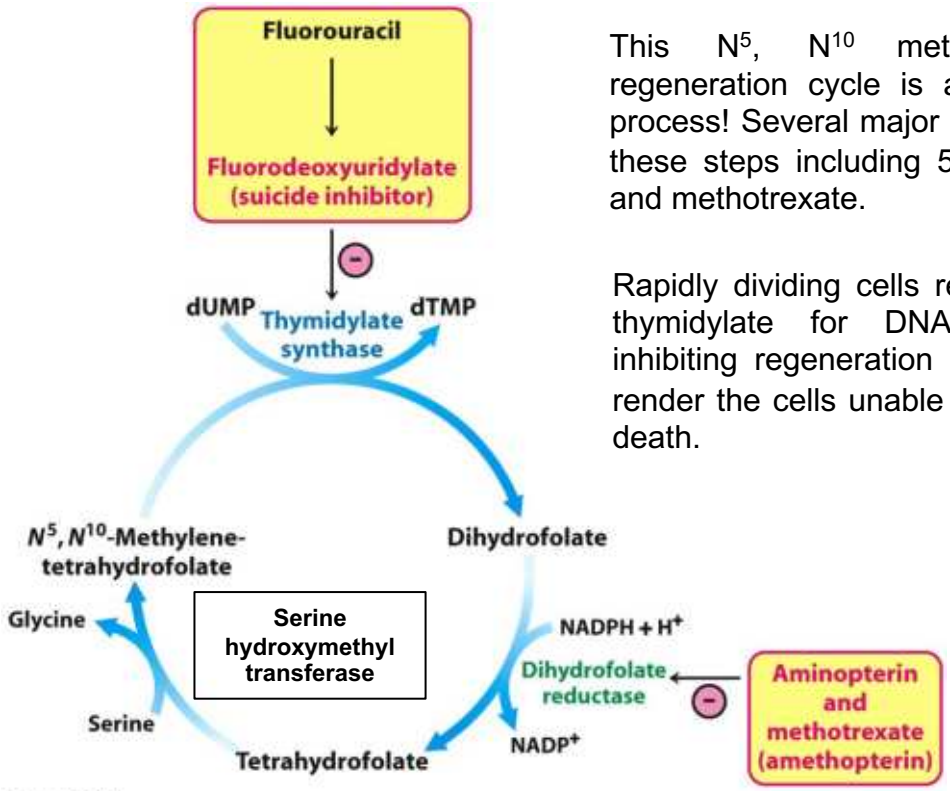
- Methyl donor is N^5,N^{10}-methylenetetrahydrofolate
- Substrate forms covalent intermediate with enzyme cysteine

Cycle to regenerate N^5, N^{10}-methylene THF

N^5, N^{10} methylene tetrahydrofolate (THF) must be regenerated after use in TMP synthesis. Thymidylate synthase removes the methylene group from N^5, N^{10} methylene tetrahydrofolate leaving dihydrofolate. First, dihydrofolate is reduced to tetrahydrofolate by dihydrofolate reductase using NADPH. The single carbon is then regenerated by serine hydroxymethyl transferase using serine as the carbon source for the methylene group and synthesizing N^5, N^{10} methylene tetrahydrofolate.



Clinical Aside: Anticancer drugs target DNA synthesis

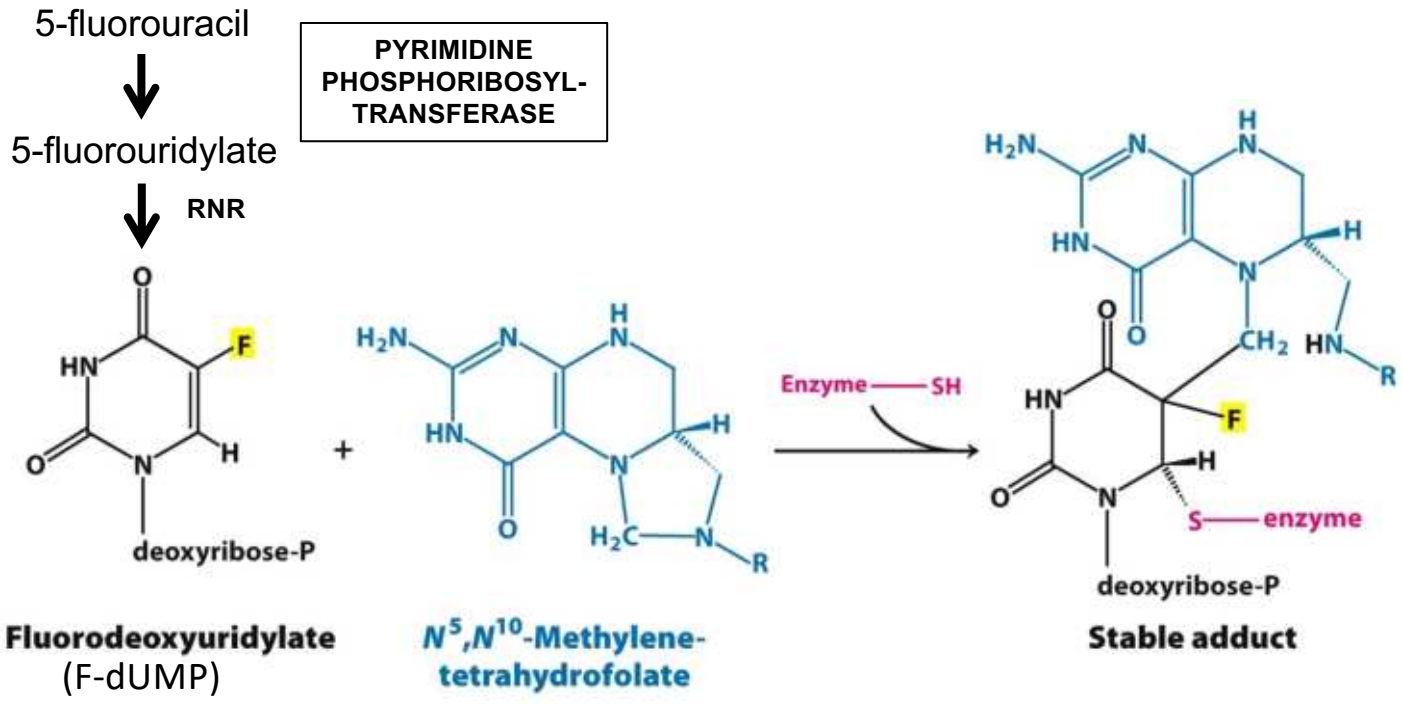


This N^5, N^{10} methylene tetrahydrofolate regeneration cycle is a very clinically relevant process! Several major chemotherapeutics target these steps including 5-fluorouracil, aminopterin and methotrexate.

Rapidly dividing cells require large quantities of thymidylate for DNA synthesis. Therefore, inhibiting regeneration of this methyl donor will render the cells unable to divide and lead to cell death.

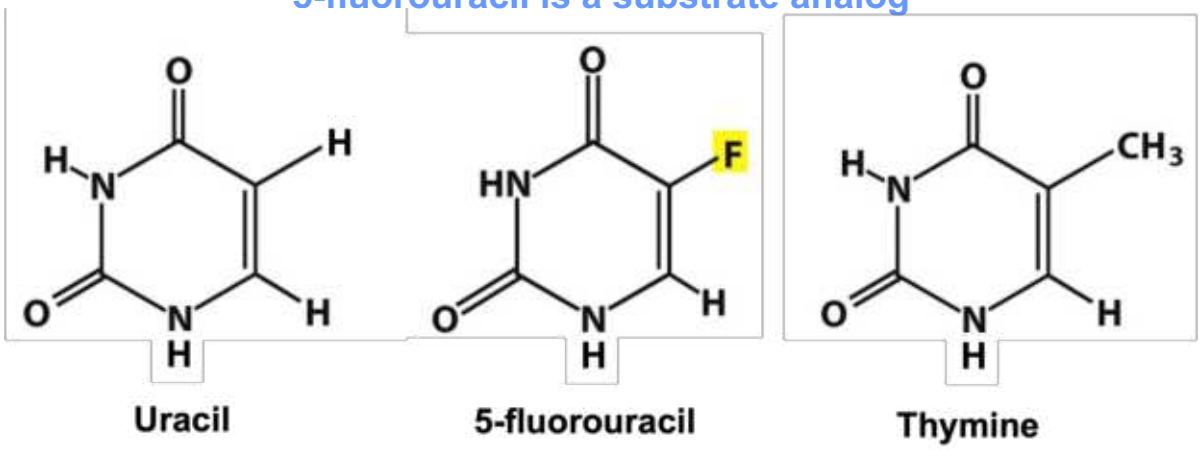
5-fluorouracil is a suicide inhibitor of thymidylate synthase

5-fluorouracil is a structural analog of the base uracil. It can be converted to 5-fluorouridylate and then reduced to the deoxy form (F-dUMP) through the same enzymes that would naturally process uracil. F-dUMP partially reacts with N^5, N^{10} methylene tetrahydrofolate and thymidylate synthase, but becomes trapped in the active site. The methylene group can not be transferred to the C5 position of the nucleoside as it would with UMP to generate thymidylate. The fluorine at the C5 position of F-dUMP blocks catalysis and renders the enzyme inactive.



Suicide inhibition: Fluorodeoxyuridylate forms a long-lived covalent intermediate with thymidylate synthase and thus blocks enzyme activity.

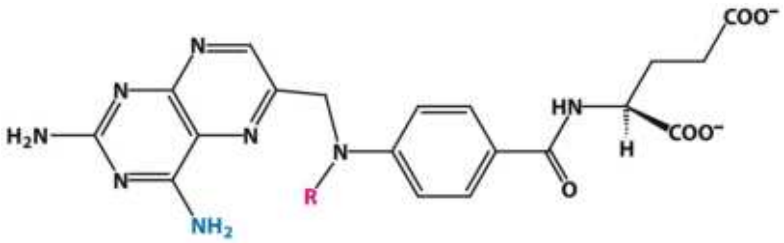
5-fluorouracil is a substrate analog



In this diagram, you can easily see how the fluorine in 5-fluorouracil will block the methylation reaction typically carried out by thymidylate synthase in the natural conversion of deoxyuridylate to thymidylate.

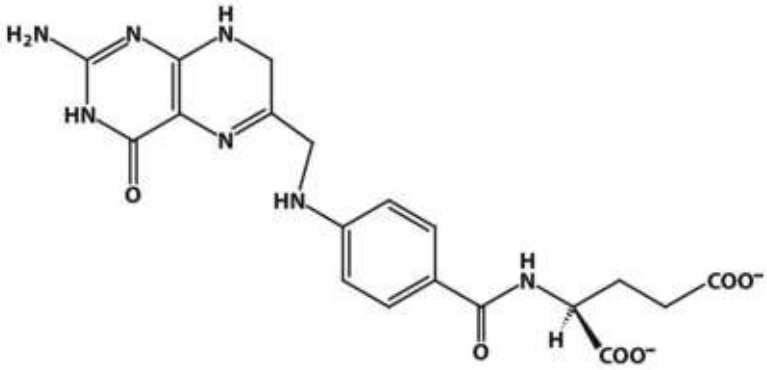
Aminopterin and methotrexate are competitive inhibitors of dihydrofolate reductase (DHFR)

5-FU inhibits thymidylate synthase directly. Aminopterin and methotrexate target dihydrofolate reductase which inhibits the cell's ability to regenerate N^5, N^{10} methylene tetrahydrofolate, consequently inhibiting the thymidylate synthase reaction as well. Dihydrofolate reductase reduces dihydrofolate to tetrahydrofolate. Aminopterin and methotrexate are structural analogs of dihydrofolate. They both act as competitive inhibitors, binding in place of dihydrofolate to block the active site. Both drugs are used as chemotherapies as they affect rapidly dividing cells efficiently. Methotrexate is also a DMARD used for inflammatory diseases.



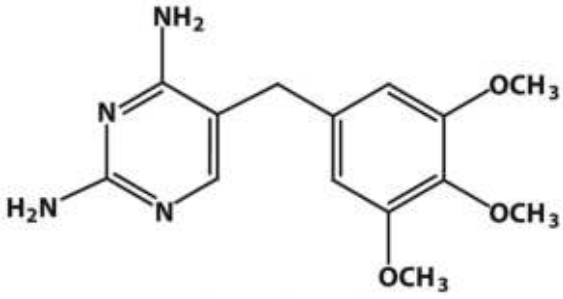
Aminopterin (R = H) or methotrexate (R = CH_3)

k_i < 1nM



Dihydrofolate

Anti-folates can have antibacterial and antiprotozoal activity



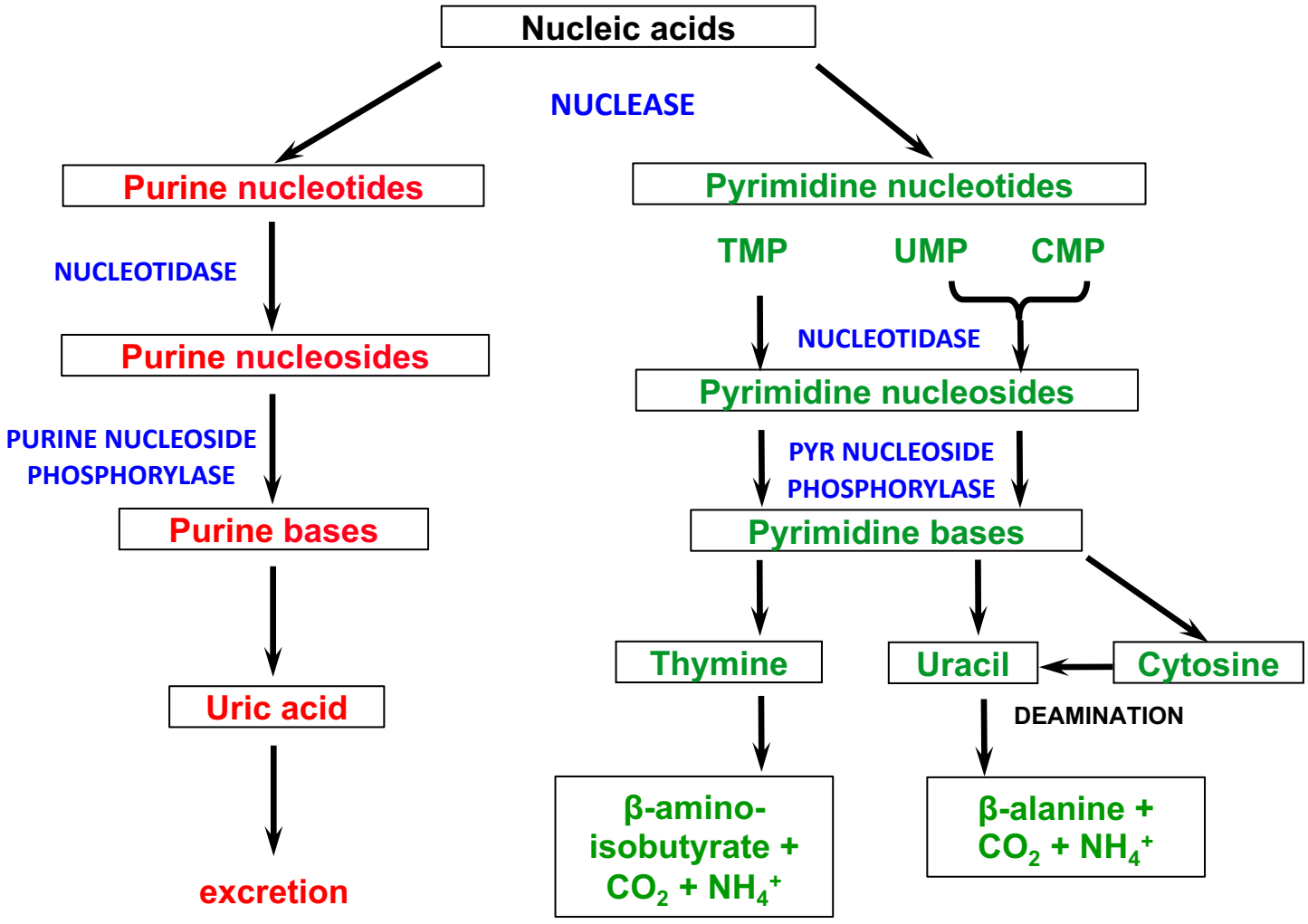
Trimethoprim

- Inhibitor of bacterial DHFR
- Binds 10^5-fold less tightly to human DHFR

Another DHFR inhibitor is trimethoprim, which binds specifically to the bacterial DHFR enzyme and has a much lower affinity for the human enzyme. Trimethoprim is one component of the combination therapy Bactrim, a drug that we discuss in the pentose phosphate pathway lecture as well. Pyrimethamine binds protozoal DHFR and is used as an anti-parasitic agent.

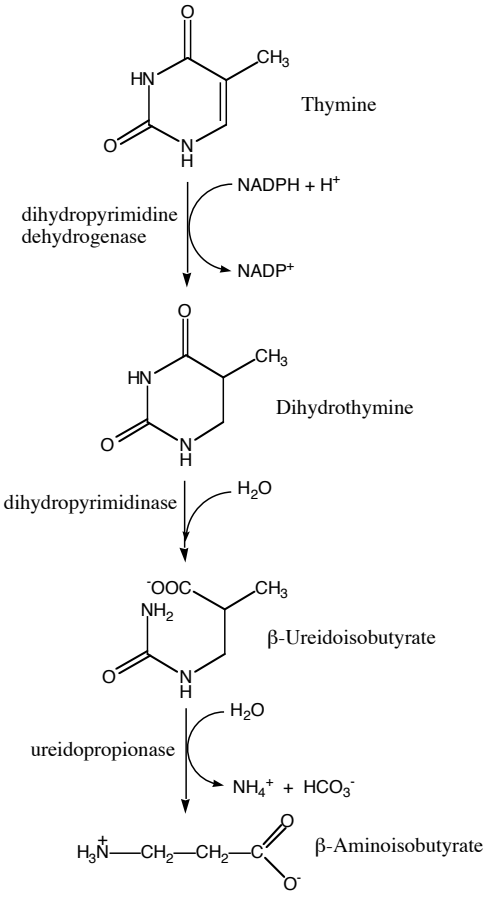
4. Breakdown of nucleotides

The diagram below depicts an overview of the breakdown pathways for both purines and pyrimidines. Both cascades begin with dephosphorylation by nucleotidases and then removal of the ribose moiety to generate bases using either purine or pyrimidine nucleoside phosphorylase. Further detail on the purine breakdown pathway is shown on a later slide. Thymine breakdown is also shown on the next slide. The simple breakdown of cytosine and uracil are shown below.



4a. Breakdown of thymine

Thymine is broken down to β-Aminoisobutyrate. **β-Aminoisobutyrate** is excreted in urine of humans and originates exclusively from degradation of thymine.

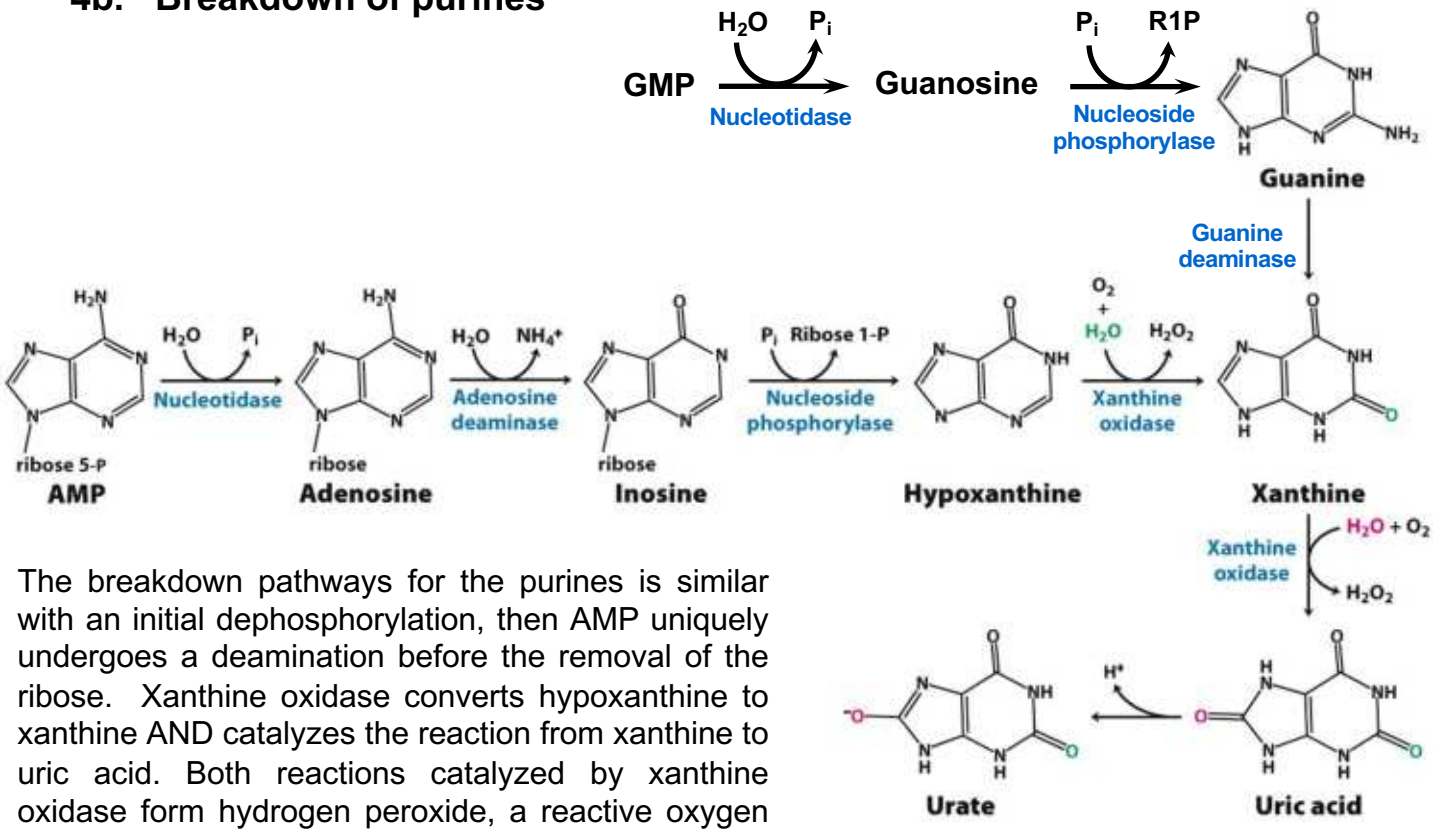


Clinical aside:
Monitoring Chemotherapy

•Increased levels of β-aminoisobutyrate are present in urine from cancer patients undergoing chemotherapy or radiation therapy due to destruction of the tumor cells.

•These patients also show increased serum and urine concentrations of uric acid (from purine breakdown).

4b. Breakdown of purines



The breakdown pathways for the purines is similar with an initial dephosphorylation, then AMP uniquely undergoes a deamination before the removal of the ribose. Xanthine oxidase converts hypoxanthine to xanthine AND catalyzes the reaction from xanthine to uric acid. Both reactions catalyzed by xanthine oxidase form hydrogen peroxide, a reactive oxygen species. Uric acid, and the charged form urate, are excreted in the urine.

4c. Nucleotide breakdown clinical asides:

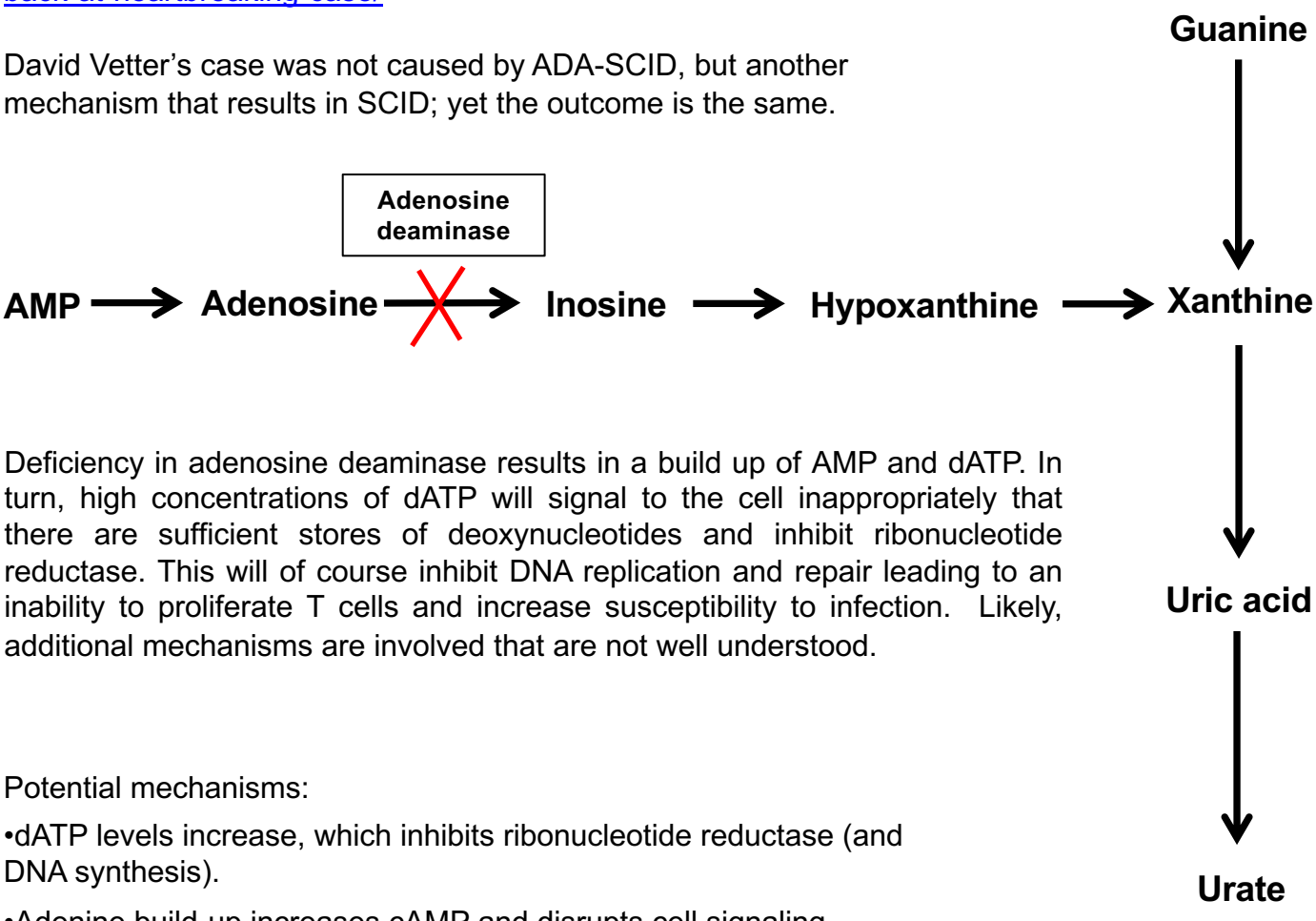
i. Deficiency of adenosine deaminase (ADA)

ADA-SCID

- One cause of severe combined immunodeficiency (SCID)
- Autosomal, recessive, 1 in 100,000 live births
- Symptoms are recurring infections; patients often die at an early age
- Immunodeficiency is due to loss of T cells
- Treatments: bone marrow transplantation, enzyme-replacement therapy, gene therapy and stem cells.
- Strimvelis is *ex vivo* stem cell treatment approved in 2016
- Or complete isolation in sterile environment

David Vetter – the original "bubble boy":
http://www.cbsnews.com/pictures/bubble-boy-40-years-later-look-back-at-heartbreaking-case/

David Vetter's case was not caused by ADA-SCID, but another mechanism that results in SCID; yet the outcome is the same.



Deficiency in adenosine deaminase results in a build up of AMP and dATP. In turn, high concentrations of dATP will signal to the cell inappropriately that there are sufficient stores of deoxynucleotides and inhibit ribonucleotide reductase. This will of course inhibit DNA replication and repair leading to an inability to proliferate T cells and increase susceptibility to infection. Likely, additional mechanisms are involved that are not well understood.

Potential mechanisms:

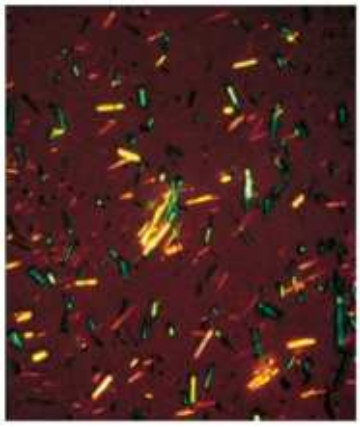
- dATP levels increase, which inhibits ribonucleotide reductase (and DNA synthesis).
- Adenine build-up increases cAMP and disrupts cell signaling

ii. Gout/ Allopurinol

INTO THE CLINIC

Lotta Topaigne

- 45-year old woman
- Complains of a severe throbbing pain in the right big toe
- Toe shows no trauma, but appears red and swollen
- Toe is exquisitely tender to even light pressure
- Passive motion of the joint causes pain



Joints and kidneys are damaged by sodium urate crystals in gout.

Gout

•*Gout* is a form of arthritis caused by *hyperuricemia*.

•Affects 1% of population in Western countries

•9 times more common in men than women

•*Hyperuricemia* is defined as a plasma urate (uric acid) level greater than 7.0 mg/dL

•Sodium urate crystals in joints cause inflammation, arthritis and PAIN, can also develop uric acid kidney stones in the kidneys

Treatment is **allopurinol** – start therapy several weeks after the acute gout attack has subsided

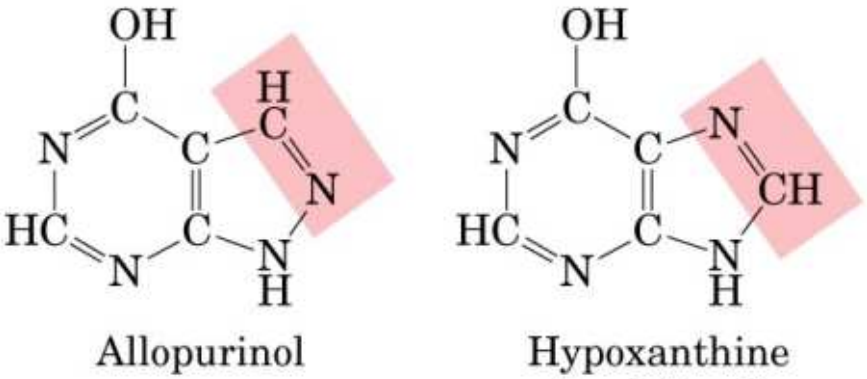
Long term treatment for gout is allopurinol which blocks purine breakdown and uric acid formation by blocking xanthine oxidase. In the short term, lithotrophy can be used to disrupt kidney stones and anti-inflammatory glucocorticoids can be used.

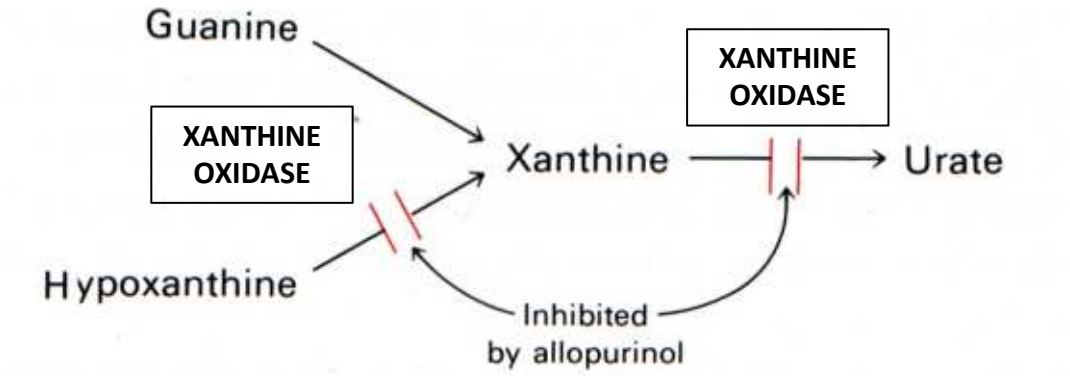
Gertrude Elion: 1988 Nobel prize in physiology and medicine

Involved in the first purine analog drug synthesis and other drugs listed below at Burroughs Wellcome.

- Allopurinol (gout)
- Mercaptopurine (leukemia and organ transplant)
- Acyclovir (viral herpes)
- Azidothymidine (HIV)

Allopurinol





Allopurinol, also known as Zyloprim, is a hypoxanthine analog and therefore a competitive inhibitor for xanthine oxidase preventing build up of urate. Febuxostat (aka Uloric) is a recently introduced second-line treatment for gout for those that cannot tolerate allopurinol. Same mechanism of action but it is more selective for xanthine oxidase.

Those predisposed to gout can reduce their intake of purine rich food, including red meat, game, seafood (especially mussels, herrings and sardines) and alcoholic drinks, particularly beer. Dietary adjustments can be effective for some individuals, but not all.

purine table and information

HIGHEST IN PURINES (400 mg. uric acid/100 g and higher)				
Foods (alphabetically)	**Total Purines in mg uric acid/100 g (Average)**	**Min**	**Max**	**Nutrition Density in mg/MJ**
Fish, sardines in oil	480	399	560	519.5
Liver, Calf's	460			837.5
Mushroom, flat, edible Boletus, dried	488			932.8
Neck sweet bread, Calf's	1260			3012.9
Ox liver	554			1013.3
Ox spleen	444			1052.6
Pig's heart	530			1382
Pig's liver	515			937.9
Pig's lungs (lights)	434			911.2
Pig's spleen	516			1208.2
Sheep's spleen	773			1702.6
Sprat, smoked	804			795.6
Theobromine	2300			1611.3
Yeast, Baker's	680			2071.3
Yeast, Brewer's	1810			1866.6
MODERATELY HIGH IN PURINES (100 to 400 mg. uric acid/100g)				
Foods (alphabetically)	**Total Purines in mg uric acid/100 g (Average)**	**Min**	**Max**	**Nutr. Density in mg/MJ**
Bean, seed, white, dry	128			127.1
Bean, Soya, seed, dry	190			139.1
Beef, chuck	120			192
Beef, fillet	110			216.4
Beef, fore rib, entrecote	120			185.4

http://www.n1health.com/Media/Corporate/McHenry/Documents/Purine%20Table.pdf

5. Salvage pathways

The salvage pathway simply uses free bases from the bloodstream derived from the diet (small percentage) or from turnover of nucleotides (especially from liver). Most of our cells can salvage these bases from the bloodstream for RNA and DNA synthesis by attaching an activated ribose molecule in the form of PRPP.

5a. Purine and pyrimidine salvage pathway enzymes

Purines

adenine + PRPP ⟶ adenylate + PP_i — **ADENINE PHOSPHORIBOSYL-TRANSFERASE (APRT)**

guanine + PRPP ⟶ guanylate + PP_i

hypoxanthine + PRPP ⟶ inosinate + PP_i — **HYPOXANTHINE-GUANINE PHOSPHORIBOSYL-TRANSFERASE (HGPRT)**

↖ Results from breakdown of adenine

Two enzymes function in the purine salvage pathway (Adenine phosphoribosyl-transferase (APRT) to make adenylate and hypoxanthine-guanine phosphoribosyl-transferase (HGPRT) to make guanylate and inosinate, the purine precursor nucleotide. Remember that we have seen "PRT" enzymes before that always hydrolyze the pyrophosphate of a PRPP molecule to drive addition of ribose.

Pyrimidines

uracil + PRPP ⟶ uridylate + PP_i

cytosine + PRPP ⟶ cytidylate + PP_i — **PYRIMIDINE PHOSPHORIBOSYL-TRANSFERASE**

(relatively non-specific - higher affinity for uracil than cytosine and none for thymine)

thymine + deoxyribose 1-phosphate ⟶ thymidine + P_i — **THYMINE PHOSPHORYLASE**

Pyrimidine phosphoribosyl-transferase has no affinity for thymine. A separate reaction scheme has evolved for thymine using thymine phosphorylase. Instead of using PRPP, deoxyribose-1-phosphate is added to thymine to generate thymidine.

5b. Genetic defects in salvage pathway enzymes

Shortening of distal portions of several fingers

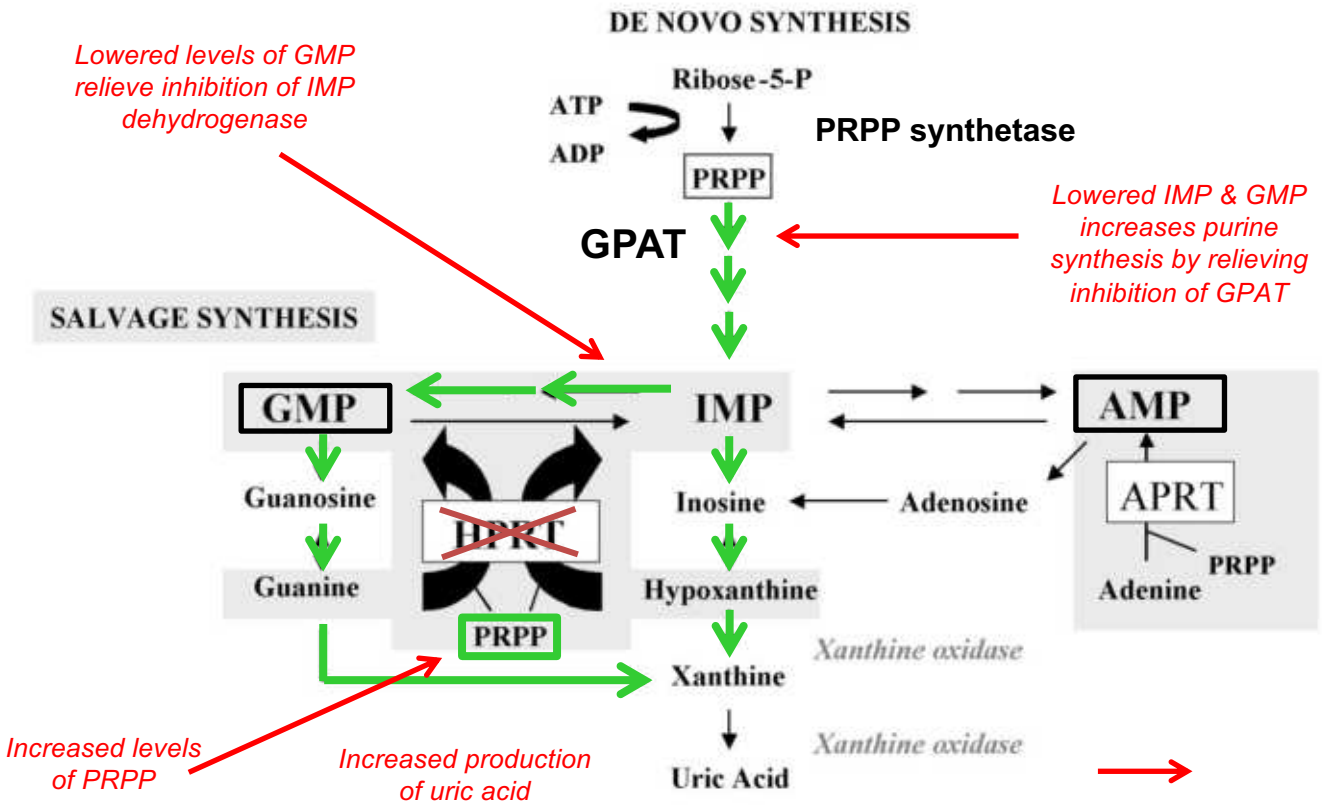
A portion of the lower lip is disfigured by persistent self-biting

Clinical Aside: Lesch-Nyhan Syndrome (LNS)

- Rare, inherited disorder caused by **deficiency of HGPRT**
- X-linked, recessive
- Frequency: 1 per 380,000 live births (in the U.S.)
- Mostly afflicts males. Females generally asymptomatic except for increased risk of gouty arthritis because it is X-linked.
- Causes a build-up of uric acid in body fluids, leading to hyperuricemia and hyperuricosuria, kidney stones and gout
- Neurologic dysfunction: spasticity, mental retardation, and behavioral disturbances, including compulsive self injury and hostile behavior.
- Severity of disease depends on specific mutations (loss of protein, shorter half-life of protein, lowered activity of enzyme)
- Allopurinol used to treat gout symptoms, lithotripsy for kidney stones
- No treatment for neurological symptoms
- Prognosis is poor – patients usually die within first or second decade of life

Mechanism of uric acid build up in Lesch-Nyhan Syndrome is shown on the next page.

Perturbation of purine metabolism in LNS



Adapted from Torres et al (2007). Orphanet *J. Rare Dis.* **2**, 1

As in LNS, a defect in the salvage pathway enzyme HGPRT will render the system unable to efficiently generate nucleotides from free guanine and hypoxanthine, thereby pushing the pathway towards breakdown of these bases. Uric acid build up will lead to the gout symptoms associated with this syndrome. Exacerbation of this pathway occurs as IMP and GMP are degraded, the inhibitory mechanisms on GPAT and IMP dehydrogenase are relieved, increasing nucleotide synthesis. These newly synthesized nucleotides will end up going through breakdown as well, because HGPRT is deficient, and will increase uric acid build up. The mechanism of action for the severe neurological symptoms is not well understood.

Two additional genetic diseases associated with the purine synthesis pathway are shown below.

Kelley-Seegmiller Syndrome

•Caused by partial deficiency of HGPRT
•Causes gout and kidney stones
•Less severe than LNS – less neurological involvement

Hyperactivity mutants of PRPP synthetase

•Rare disorder, X-linked
•Severe and mild forms
•Increased activity of PRPP - activates GPAT
•Causes hyperuricemia, hyperuricosuria and gout
•Treatments: allopurinol/febuxostat, plus low purine diet

Both defects increase *de novo* purine synthesis

Summary

•Nucleotides serve important functions (RNA, DNA, cofactors, signal transduction, energy metabolism).

•Two pathways for biosynthesis: *de novo* and salvage

•PRPP is a precursor for both pathways.

•In *de novo* pyrimidine synthesis, ring is built, then R5P is added.

•In *de novo* purine synthesis, purine is built onto the R5P.

•Salvage pathways add R5P to free bases.

•Deoxyribonucleotides are made by reduction of ribonucleotides (NDPs).

•TMP is formed by methylation of dUMP.

•Several anticancer, immunosuppressive and antimicrobial drugs target nucleotide synthesis pathways.

•Key steps in nucleotide biosynthesis are regulated by feedback inhibition [CPS-II for pyrimidines and GPAT for purines]

•Uric acid is the end product of purine degradation and its accumulation as urate crystals in joints results in gout.

•Enzyme deficiencies in nucleotide metabolism cause diseases, *e.g.* LNS and ADA-SCID.