Nitrogen Fixation

One of the wonderful things about plants are their ability to take starting materials that we don’t want, or can’t use in their current form, and convert them into materials that we do want or that we can use. For example, we know that plants take in gaseous carbon dioxide and use the carbon in the carbon dioxide to make glucose. There is an overload of oxygen from the carbon dioxide which the plants excrete as molecular oxygen. The plants take the unwanted carbon dioxide and make it into sugar and oxygen, items that we do want and need.

Some plants also work with nitrogen. In particular, there are a number of strains of bacteria, either free in soil or associated with the root systems of leguminous plants (beans, alfalfa, etc.) that can take gaseous nitrogen out of the environment and convert it into ammonia. The conversion of nitrogen to ammonia is the terminus of the electron transport chain in these anaerobic bacteria, equivalent to the terminal reduction of oxygen to water in aerobic organisms such as ourselves. The ammonia is either oxidized to nitrate or released into the environment as ammonia.

Three dimensional structures of the nitrogenase enzyme complex – the one involved in nitrogen fixation – have been determined. The nitrogenase is an iron-molybdenum protein, where the iron is involved in electron shuttling and the actual nitrogen reduction takes place on the molybdenum. Nitrogen reduction requires eight electrons and eight protons, with a reaction by-product of one molecule of hydrogen gas.

If any of you have any farming blood, traditional farmers use crop rotation in order to replenish nutrients in their fields. Typically, for two growing seasons a grain crop would be planted in a field, and then in the third, a leguminous plant such as alfalfa or soybeans would be planted. The bacteria associated with the roots of these plants will replenish the soil with nitrates, which would be depleted by the grain plants. The plants could be harvested at the end of the season, but are often plowed back into the soil to provide additional nutrients.

Nitrogen oxidation

The reduction of nitrate to ammonia is common to most plants and bacteria. Nitrate is reduced to ammonia in four steps. The first step involves the enzyme nitrate reductase, while the last three are carried out sequentially by nitrite reductase. The sequence of steps goes:

\[ \text{NO}_3 \rightarrow \text{NO}_2 \rightarrow \text{NO} \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NH}_3 \]
**Digestion of proteins**

As you well know, we are omniverous animals - we are capable of digesting both delicious vegetables and the dead carcasses of other animals. We, in fact, prefer to eat the dead carcasses of other animals over the delicious vegetables. Plants have the wonderful ability to store energy as primarily complex carbohydrate, which our bodies can use just fine. Do you know that the adult male need the protein equivalent of 1 egg a day to meet its minimum daily requirement? But we still prefer to eat the charred remains of other species.

In fact, some of the great nutritionists of our day, including Dr. Marsh Morrison, recommend against a high protein diet. Someone had given me a copy of his book to look over; it was published by a “vanity” house. He had some unorthodox views on the evils of the protein diet. For example, he stated that protein is one of the major causes of aging, and especially those nasty foul-smelling stools we all have been troubled with. I eventually got tired of looking at his leering face on the front cover and threw the book away.

Anyway, we do eat protein, probably in excess of what we need most of the time. Our body, while preferring to use carbohydrate for energy, will not waste any potential energy source. There are three reasons why our bodies might catabolize amino acids to smaller carbon units, either for energetic or biosynthetic purposes:

1. We simply eat more than we need as amino acids.
2. Some organisms, especially the bean family, use protein for an energy storage medium. This is why beans are a high-protein vegetable, which is something I didn't appreciate until I wrote this lecture.
3. Metabolic turnover of endogenous proteins. Do you remember when we talked about the dietary regulation of acetyl-CoA carboxylase, how the levels of this protein decrease on fasting. How do our cells get rid of this protein? By cutting it up and metabolizing it!

Let's get back to talk a bit about protein intake and catabolism. How are proteins catabolized, and to what end? The first step in protein catabolism is breakdown to constituent amino acids. This can be used to make new proteins, or they can be broken down into small carbon compounds which can be used to make other cellular things, such as fatty acids, or be totally degraded into carbon dioxide and energy.

As we will discuss later in the class, excess amounts of most amino acids are toxic to our
bodies, so much of the time our bodies spend metabolizing amino acids is simply to eliminate excess concentrations from our cells.

As we did with fats, let's follow our steak as it disappears into our stomachs and see how the protein in the steak is digested and absorbed. In the stomach, the high acid environment and the proteolytic enzyme peptin begin to hydrolyze some of the peptide bonds in the protein. As the broken proteins enter into the small intestine, they are metabolized further into small peptides by the enzymes chymotrypsin and trypsin. The resultant small peptides are completely hydrolyzed to their amino acids by nonspecific peptidases which are associated with the intestinal lining. Single amino acids are transported across the intestine to the circulatory system, where they are transported around the body and absorbed into cells by specific amino acid transport proteins.

As far as I can discern from my sources, most of the enzymes involved in amino acid catabolism are located in the mitochondrial matrix. Also, in our bodies, most amino acid catabolism occurs in the liver. There is some catabolic activity in the kidney, but hardly any in skeletal muscle, for example. I will mention exceptions to this rule as we go, which are in general located in the urea cycle, which we will speak about soon.

The major problem our bodies have to deal with in amino acid catabolism is: what do we do with the nitrogen? Our bodies have two general mechanisms to remove nitrogen, in the form of ammonia, from amino acids; these are transamination (or aminotransferase reactions), and oxidative deamination.

An example of an aminotransferase reaction is the one carried out by glutamate aminotransferase. Some L-amino acid donates its ammonia group to α-ketoglutarate, the citric acid cycle intermediate, to form the amino acid glutamate plus the α-keto acid derivative of the original amino acid. This is a reversible reaction, requiring no input of energy. The driving force behind this reaction is concentration differences. Glutamate has the ability to lose its amine group in several different ways, as we will soon discuss.

Aminotransferases contain a tightly bound pyridoxyl-5'-phosphate cofactor, the structure of which is this.

The pyridoxyl 5'-phosphate acts as an amino carrier in the aminotransferase proteins. In
the resting state, the pyridoxyl 5′-phosphate aldehyde group is attached to a side chain lysine by a Schiff's base linkage. The first step in the enzyme reaction is displacement of the lysine by the L-amino acid to form a Schiff's base linkage between the pyridoxyl and the L-amino acid, in what is called an aldimine. This undergoes rearrangement, with deprotonation and protonation, to form the ketimine intermediate. Hydrolysis frees the α-keto acid, leaving behind the amino group as pyridoxamine phosphate. The amine group is then transferred to glutamate by the reverse of this mechanism.

Now, it's clear that the aminotransferase reaction does not lead to net loss of nitrogen. An amine group is simply moved. One amino acid is converted to an α-keto acid, but a new amino acid is made to replace it. A net deamination reaction requires a separate pathway, which, as you might have guessed, is the oxidative deamination enzyme I mentioned. The enzyme which catalyzes oxidative deamination is glutamate dehydrogenase, which catalyzes the liberation of free ammonia from glutamate with concurrent reduction of either NAD+ or NADP+:

The mechanism of glutamate dehydrogenase begins with the transfer of a hydride ion to NAD+, forming a carbon-nitrogen double bond. The carbon atom is now fairly electronegative, and susceptible to attack by an electropositive nitrogen at the terminus of an enzyme lysine side chain in what is called a transimidation reaction. This releases free ammonia, which is the goal of the reaction. The α-keto glutamate, the final product of the reaction, is released by hydrolysis of the imide by water.

Most of the time, we do not need to incorporate ammonia into carbon containing compounds – we eat more of these than we need. But if necessary, we can incorporate free ammonia to make several amino acids. First, glutamate dehydrogenase is a reversible enzyme, and can incorporate ammonia into α-ketoglutarate to make glutarate. There is a second enzyme in plants and bacteria, glutamate synthase, that carries out the same reaction but has a much higher affinity for ammonia. It works much more efficiently in the synthetic direction than glutamate dehydrogenase.

Ammonia can be added on to the side chain carboxylate group by the enzyme glutamine synthetase to make the amino acid glutamine. The enzyme requires the energy in one ATP in
order to create the amide bond:

Glutamine synthase is important in the biosynthesis of several amino acids and also nucleotides and amino sugars. It is regulated by two mechanisms: allosterically by one of eight different inhibitors, or by covalent modification. The eight inhibitors are end products of the biosynthetic pathways fed by glutamine. Any one of these agents will bind to glutamine synthetase and inhibit it. With covalent modification, tyrosine amino acids are adenylated with ATP to make a phosphoadenyl tyrosine residues. The more residues that are adenylated, the less active the protein; a completely adenylated protein is inactive.

The adenylation is well characterized in bacteria, but regulation of the mammalian enzyme is not well characterized.

**Protein Turnover**

One of the newer items covered in Mathews is the concept of protein turnover. All of the biomolecules in our cells are synthesized and degraded on a regular basis. Protein turnover is particularly important for this topic, since when proteins are broken down, our cells have to process the potentially toxic nitrogen that is produced.

Proteins are degraded in the cell into their constituent amino acids by two major mechanisms. There are cytoplasmic proteases that recognize targeted proteins and digest them into amino acids. There are two types of calpains, components of proteasomes, that degrade shorter lived intracellular proteins. Lysozomes, and the proteases found in lysozomes called cathepsins, degrade proteins taken in from outside of the cell, and longer lived cellular proteins. For example, the LDL receptors are degraded in lysozomes.

I was listening to the radio this morning and learned that a new season of the Sopranos will be coming out in March. I don’t get cable tv, and have only seen a few clips of the Sopranos, but I generally like crime/mob dramas, and think I would enjoy this. Why do I bring this up now? Imagine these

What tells these protein hit-men to take out their targets? Your textbook mentions four factors that target proteins for rapid turnover. First, our cells contain a protein called ubiquitin. This is a small, heat-stable protein found in large amounts in most eukaryotic cells. Ubiquitin reacts with a target protein by forming ester bonds between the C-terminal glycine of ubiquitin and lysine amino acid side chains on the targeted protein. The proteosome recognizes the
ubiquitin-modified protein as ready for turnover, and degrades it.

Second, protein oxidation seems to play a role in turnover. Oxidized proteins are preferentially degraded over non-oxidized proteins. Protein oxidation can occur through the intercession of oxygen radicals or metals. There is evidence that links oxidative damage to cells to the aging process, and a number of studies have indicated that the regular consumption of antioxidants is beneficial to health. Third, proteins that turnover quickly contain regions rich in the amino acids proline, glutamate, serine, and threonine. These so-called “PEST” sequences are recognized by proteolytic enzymes and cause the protein to be degraded. Fourth, the identity of the N-terminal amino acid plays a role in the half life of proteins. Proteins that contain the amino acids phe, leu, tyr, trp, lys, or arg have shorter lifetimes than proteins that begin with other amino acids. Again, mutation of the N-terminal amino acid of proteins from one of these amino acids to one not on this list increases the half life of the protein.

**Urea Cycle**

What happens to the free ammonia? Ammonia is toxic to most organisms at moderate concentrations, so our bodies have to remove this ammonia from our system. Different organisms possess different mechanisms for eliminating ammonia.

In simple organisms, mostly single cell organisms, ammonia is excreted to its environment as ammonia. In birds and terrestrial reptiles, ammonia is mostly excreted in the form of uric acid. The synthesis of uric acid in birds and reptiles is similar to the synthetic pathways for purines.

In our bodies, ammonia is excreted as urea. Urea is produced in mammals by the enzymes of the urea cycle. The urea cycle involves the coordinated efforts of 5 different enzymes, three of which are in the cytoplasm and 2 in the mitochondrial matrix. The first reaction of the urea cycle is the formation of carbamoyl phosphate by the action of the enzyme carbamoyl phosphate synthase. One carbon dioxide, one ammonium ion, and one phosphate ion donated from ATP are fused together using the energy of two ATP hydrolysis reactions. This reaction occurs in the mitochondrial matrix. The second reaction of the cycle is the condensation of the carbamoyl group of the carbamoyl phosphate with L-ornithine to yield L-citrulline, which is catalyzed by the enzyme ornithine transcarbamoylase. Inorganic phosphate is lost in the reaction; the reaction is energized by the hydrolysis of the carbon-phosphorus bond. L-citrulline
is then transported out of the mitochondrial matrix into the cytoplasm. An L-aspartate forms a Schiff's base with the carbamoyl carbonyl group, catalyzed by the enzyme arginosuccinate synthase. The enzyme arginosuccinate lyase liberates succinate from arginosuccinate, yielding L-arginine. The enzyme arginase catalyzes the removal of urea from the side chain of L-arginine, leaving the product L-ornithine. The ornithine is transported back into the mitochondrial matrix by an amino acid transport system.

The stoichiometry of the urea cycle is:

\[
\text{CO}_2 + \text{NH}_4^+ + 3 \text{ATP} + \text{aspartate} + 2 \text{H}_2\text{O} \rightarrow \text{urea} + 2 \text{ADP} + 2 \text{Pi} + \text{AMP} + \text{PPi} + \text{fumorate}.
\]

The urea formed in the cycle has 2 nitrogen atoms; one coming from the oxidative deamination of glutamate and one from the α-amino group of aspartate.

The urea cycle is linked to the citric acid cycle. Aspartate can be generated from oxaloacetic acid by an aminotransferase reaction. The arginine can then combine with citrulline to form arginosuccinate. Lysis of arginosuccinate results in fumeric, which can return to the citric acid cycle, and arginine, which can continue in the urea cycle.

The urea cycle is found in our bodies only in the liver. Ammonia must be transported from the tissues in which it is generated to the liver in order to be excreted as urea. A common ammonia transporter in our bodies is glutamine. Glutamine is synthesized from glutamate enzymatically using the enzyme glutamine synthase. The glutamine generated in extrahepatic tissues is released to the blood and taken up by the liver, where ammonia is released by the action of the enzyme glutaminase.
Skeletal muscle specifically uses alanine as a transporter of ammonia back to the liver. Alanine is formed from a transamination reaction between glutamate and pyruvate. Alanine returns to the liver and donates its nitrogen back to α-ketoglutarate to make glutamate.

**Cofactors involved in Nitrogen Metabolism**

There are three cofactors that are important in nitrogen metabolism. The first is pyridoxyl phosphate. We discussed pyridoxyl phosphate earlier in the class when we examined the mechanism of the transaminase reaction.

The second important cofactor in amino acid metabolism is tetrahydrofolate. Tetrahydrofolate is an important coenzyme in our bodies. It is derived from the vitamin folic acid. It is reduced to tetrahydrofolate in two steps, both using the enzyme dihydrofolate reductase.

Dihydrofolate reductase is a clinically important target for anticancer drugs. Cancerous tumors perform a great deal of biosynthetic reactions (in particular, nucleotide synthesis), and require many reactions that transfer carbon atoms on and off of tetrahydrofolate. If the formation of tetrahydrofolate from folic acid is blocked, then the growth of the tumor cells is greatly reduced. Aminopterin and methotrexate are two drugs that are effective against certain types of cancers. Other rapidly dividing organisms such as bacteria and parasites are also susceptible to treatment by inhibition of their dihydrofolate reductases. Trimethoprim is an antibiotic that inhibits DHRF, while pyrimethamine targets the same enzyme in protozoa.

We discussed S-adenosylmethionine as a methyl group donor. It carries methyl groups and acts as a methyl group donor to a number of reactions, including the phosphatidycholine biosynthesis we discussed earlier in the class. Tetrahydrofolate can act as a carrier of one-carbon units in several different oxidation states. To describe what I mean, tetrahydrofolate can essentially either donate or accept a molecule of methanol (lowest oxidation state), formaldehyde (intermediate oxidation state), or formic acid (highest oxidation state). It can transfer a methyl group, when it does it is carried on the tetrahydrofolate as N-5 methyl (where the ring nomenclature shown below). It can donate a methylene group, which is more oxidized than the methyl group; the carbon atom is bound to tetrahydrofolate as N-5, N-10 methylene tetrahydrofolate. It can be bound in its highest oxidation state either as N-5 formyl, N-10 formyl, N-5, N-10 methenyl, or N-5 formimino.

In addition, all of these molecules are interconvertible. The interconversions between
different species of methylated tetrahydrofolate are enzyme catalyzed, and without enzymes each individual species is stable.

Now that we know the identity of all of these different tetrahydrofolate species, the next step is to identify how these are synthesized (or, which reactions require tetrahydrofolate as a methyl acceptor), and to identify which reactions require tetrahydrofolate as a methyl donor.

Many of the biosynthetic reactions in our bodies require S-adenosylmethionine as a methyl donor. Therefore, one of the most important reactions carried out using tetrahydrofolate is the regeneration of methionine from homocysteine, which is the end product after methyl transfer by S-adenosylmethionine. The enzyme which catalyzes this process is homocysteine methyltransferase, and the tetrahydrofolate species used is N-5 methyltetrahydrofolate: The enzyme uses as a coenzyme the methylcobalamin group from vitamin B_{12}. There are only two B_{12} enzymes that are important in mammals: homocysteine methyltransferase (or methionine synthase), and methylmalonyl-CoA mutase. B_{12} has a very complex heme structure with a central cobalt ion. The structure is provided on the overhead. The enzyme snitches a methyl group from N-5 methyl tetrahydrofolate and converts the B_{12} into methyl cobamalin; the methyl group replaces the deoxyadenosine group shown on the overhead. This group takes the methyl group from tetrahydrofolate, creating a methylated coenzyme intermediate, and then transfers it to homoserine.

The methyl group on tetrahydrofolate can be acquired in many ways. Two I'd like to mention are the addition of a formic acid molecule, with the energy of an ATP, using the enzyme 10-formyltetrahydrofolate synthase, to make N-10 formyl tetrahydrofolate. This can then be enzymatically converted to N-5 methyltetrahydrofolate.

Another reaction which results in methylation of tetrahydrofolate are the two reactions which convert serine to glycine, and then glycine to carbon dioxide and ammonia. Both the enzymes serine hydroxymethyltransferase and glycine oxidase add a methylene group (in the first case, initially methanol, in the second case, initially a methylene group) to tetrahydrofolate as N-5, N-10 methylene tetrahydrofolate.