JOANNE STUBBE: We're talking about the purine biosynthetic pathway. Here's the pathway. I told you, in this part of it we were going to go through, so at least you saw what the steps in the pathway are. The key thing is you start out with the ribose 5-phosphate, and then you build up the base a step at a time, which is completely different from pyrimidines, where you make the base, and then stick on the ribose 5-phosphate.

And I told you at the very beginning, there were a few interesting steps in this pathway that are universal in almost all metabolic pathways. And one of them we were going over-- two of them we already went over. I'm going to briefly go back over this, but the role of glutamine in the purine and pyrimidine pathway as the source of nitrogen. There were five of these enzymes. That's not an accident. Glutamine is one of the major ways you deliver ammonia into molecules. And purines and pyrimidines both have a lot of nitrogens.

The second thing we were talking about, and we had gone through the first few steps here, was the second enzyme in the pathway, where we use ATP, and in this particular pathway, this is the mammalian version of the pathway, which is pretty similar to the bacterial, but there were five different steps that require ATP. This pathway demonstrates how you see ATP use over and over and over and over again. There are defined structures for the binding sites of the ATPs. Once you have these in your brain, it becomes easy. You might not know which one of these mechanisms it is, but after you do a little bit of reading, or bioinformatics, you can immediately tell what the structure of the enzymes actually are. The other thing we talked about already was the role of folate.

Those are the three things I want you to get out of this, and we're going to go through the rest of that today, and then, after we finish that, we'll come back to the purinosomes, which is the reason I chose this topic a long time ago, because it speaks to the question of the importance of transient protein-protein interactions in metabolism inside the cell, which has been something that people have been interested in for decades, and this paper in 2008 that you read for recitation was very interesting to a lot of people, and we'll come back and talk about

that at the end.

The first enzyme-- the names are horrible. I gave you the names of all these things. If you look at last year's exam, you will have the purine pathway with the name stuck at the end. I don't expect you to remember this, but we go from PRPP-- we've already gone through this step-- and the enzyme is PurF-- I'm not going to write it out-- goes to PRA. The reason I'm writing that again is because a key reason that Bankovic's lab and my lab, many years ago, was focused on this is because of the instability of the intermediates in this pathway. This guy has a half life of 15 seconds at 37 degrees, so this is chemically unstable.

This is enzyme 1, and this is the first place we saw glutamine going to glutamate as the source of ammonia. And I wanted to go back and say one more thing about that. Again, there are two enzymes that use glutamine as a source of ammonia. This one is simply, if you look at the pathway displacing pyrophosphate ammonia, you have a nucleophile displacing pyrophosphate which, when complexed to magnesium, is a good leaving group. The idea here is that all of these proteins, and there were five of them, in the purine and pyrimidine pathway, have two domains. Sometimes the domains are separate polypeptides. Often they're linked together.

The glutaminase domain is in one of these domains, and the way the chemistry goes, the way the ammonia is going to displace whatever the leaving group is in the second domain, requiring a tunnel that varies from 25 to 40 angstroms to actually mediate ammonia release. PurL is the fourth enzyme in the pathway. Again, here's the glutaminase domain. It's upside down, and here's where the chemistry occurs in the other system. What I wanted to say about that is that all of these enzymes in the active site have a cysteine. All of these enzymes have a cysteine in the active site, and you should go back and look at the PowerPoint, because I'm not going to write this out on the board.

You've seen this chemistry now, over and over again, but, in some way, the glutamine is going to be attached covalently with loss of ammonia to a cysteine in the active site. Let me show you what the mechanism of that is. Here is a generic mechanism, but it could be a cysteine protease. These are the same things we've seen over and over again, so this should now be part of your basic vocabulary. So the goal, then-- here's our glutamine-- is simply to liberate ammonia.

The cysteine needs to be activated somehow for nucleophilic attack. How is that done,

normally? With a histamine. This particular enzyme. There are two superfamilies of enzymes that do this. This one doesn't use histamine, but it still needs to be activated. You go through a tetrahedral transition state, which collapses to form an acylated enzyme, and, in the end, you need to hydrolyze this off to give you a glutamic acid.

One of the reasons I wanted to go back to this, again, is because, in the Bankovic paper, we talked about, but didn't go through in any detail, the fact that, in that paper, to study whether these purinosomes could assemble and disassemble, they use an inhibitor of the purine pathway, which then should want the enzymes to assemble, because they need to make purines because you've blocked the pathway. And the inhibitor they used is a molecule that looks like this. They used azaserine, but it has another methylene in it. This is DON. And this is a diazoketone. This is a natural product, and it was discovered by Buchanan's lab at MIT, and it was the first diazo compound that people had seen. And it inhibits all-- this is something that's important when thinking about what's happening when you're treating cells with it to stop purine metabolism-- it inhibits all glutamine-requiring enzymes, because the mechanisms are similar.

So the mechanism, if you sit down and think about it, is pretty simple. You have a diazo group, and now the proposal is that this needs to be protonated by the cysteine in the active site. And now you have an N2 to that's dying to leave, N2+, and so you just do an SN2 reaction forming a covalent bond. That's the basis for how azaserine in the Bankovic paper works. There was another way that they block the pathway, which hopefully we'll have time to come back to in the end. So, again, this idea of coming together and going apart-- how do you perturb this? One way they perturbed it was depletion of purines. We discussed that. We didn't really discuss this particular step.

The next step in this pathway. Now we have R, which is ribose 5-phosphate. I'm not going to write that out, because every single step now has ribose 5-phosphate as a scaffold. And what we added was glycine. Again, here's the first time that we need to use ATP and Pi. Lots of times, you don't know, when you look at this, whether you're going to transfer pyrophosphate or you're going to phosphorylate, so where you have attack on your ATP. Almost all the enzymes, but not all of them, in the period pathway have ATP going to ADP, so that tells you the attack has to be on the gamma position.

This is an ATP grasp superfamily member, and they all go through the same mechanism, which I briefly talked about last time, so I'm not going to write this out again, but basically,

you're going to go through a phosphoanhydride, which is then attacked by a nucleophile.

We're converting the hydroxyl group of the carboxylic acid into a good leaving group. You've seen this used over and over again over the course of the semester. But over here, this is all written out for you. Here we have glycine. R is CH2NH2. You phosphorylate to form the anhydride. You still need to neutralize this to make it into a good leaving group, which is done in the active site, and then you can have a variety of nucleophiles that could come in and attack to form the covalent linkage. In this case, the nucleophile is not the NH3+. It needs to be converted to the NH2-- Sorry. The nucleophile is over here. It's phosphoribosylamine. So it's the NH2 of the phosphoribosylamine that's attacking.

Again, to be a nucleophile, it's got to be deprotonated. Hopefully, you all know that at this stage. So what do these enzymes look like? They all look the same. It turns out that if you look at, globally, purine biosynthesis, not just focus on mammalian systems, there are four or five enzymes that actually are ATP grasp superfamily members in the purine pathway. And they all look like this. They have a little domain with a lid, and all the chemistry happens in between, and the lid opens and closes. You can pick these out by bioinformatics.

That's the second step in the pathway. And this just shows what all of the products can be, so if you go back and you pull out the pathway, there are ATP grasp superfamily members, and these are the products that are formed by this common type of mechanism through a phosphoanhydride.

The next step in the pathway. So now we formed-- The next step in this pathway, let's see if I put this. All right. Sorry. I thought I put another copy of this in. The next step in the pathway is we need to formylate. What do we use as formylation? That's why we spent the introductory part of this course talking about folates, which can transfer carbon at three different oxidation levels. What you have here is, and I'm not going to draw the whole thing out, this is the part I told you was the business end.

This is N10-formyltetrahydrofolate. Theoretically, this could be either here or here, and chemically they can actually interconvert under certain kinds of conditions. But we know, for all purine pathways that people have looked at, it's always the N10. That's distinct from methylation, where it's always from the N5. I don't know how things evolved, but that's what the results are.

How does this happen? Hopefully, you all know this without me having to write this down, but

this needs to be a nucleophile. It needs to be deprotonated. You need a base to remove a proton, and then you form a tetrahedral adduct, and then the tetrahedral adduct high energy intermediate collapses, and the formyl group gets transferred from here to here.

This then becomes a molecule that looks like that. I've just transferred the formyl group, which is called FGAR. Formylglycinamide ribonucleotide. Horrible names. This molecule is unstable. It loses its formyl group actually quite rapidly. It took them a long time to figure this out.

One of the premises is a purine pathway, because people were interested in it, is that it falls apart. When you're trying to look at metabolomics, which is the next decade-- hundreds of people are using mass spec, which you guys have thought about, to look for metabolites-- you need to know something about the stability of the molecules you're looking for, and how you separate them from everything else. So this is going to be a major focus, and most people haven't found very many intermediates in this pathway, and I guarantee you it's because they break down. I think that was clear from Buchanan's work really early on.

The next enzyme in the pathway. We've seen this, again, before. Now we're going from an amide to an amidine. That's all we're doing, so an oxygen is being replaced by ammonia. So what are we going to use? We use glutamine. The next enzyme in the pathway uses glutamine to glutamate, and again, this is the source of ammonia. As I showed you before, there's a channel where this happens. This is another way you can use ATP going to ADP and Pi. This is the second kind of mechanism. This enzyme is called PurL.

Anyhow, we're using ATP again. Why are we using ATP in this case? What we're trying to do is convert this amide into an amidine. We're converting this into this. So we need a source of ammonia. That's the source of ammonia. What we have is, we're using ATP to facilitate a dehydration reaction. Again, you've seen this before with a carboxylic acid. Now we're doing it with the oxygen of the amide. The ATP is used to remove oxygen of the amide. What I'm going to show you, and then we'll come back to this again, is the generic mechanism for this. Let me show you now, before we move on, the next enzyme in the pathway. Here is using glutamine, and we use ATP to help us attach the glutamine to the carbonyl.

The next enzyme in the pathway. What you're doing, basically, I'll show you this in a second, but you're just cyclizing. This amino group becomes this amino group, and this guy has to attack that position. That position, again, is an amide, and the mechanism, again, uses ATP, just like this enzyme, PurL, and I'm going to show you how it works. These two enzymes in the

pathway are structurally homologous to each other. The product of one enzyme is the substrate for the next enzyme in the pathway, and they clearly evolve from each other. This is something that everybody's been interested in. How can you tell something about the evolution of a biosynthetic pathway, and thinking about how to control this. Why? Because everybody and his brother now is focused on bioengineering of metabolic pathways. So the more you understand about the basic principles of how nature designed this, the better off you're going to be in trying to get this to happen robustly and control things by using an enzymatic system and enzymes from many different sources.

So what's the generic mechanism? This is called-- this enzyme is part of the PurM-- the nomenclature is horrible-- superfamily. So I just told you this ATP was the ATP grasp superfamily. This is the PurM. Why is it called the PurM superfamily? Because it was the first structure of any molecule that looked like this, and it was the PurM enzyme. So that's where the horrible name came from. This enzyme is PurL, and this enzyme is PurN, and they're structurally homologous to each other. How do they work? Again, I think once you see it. Here's the general mechanism.

Here we have our amide, and what we want to do is facilitate dehydration of the oxygen. What you're going to do is phosphorylate the oxygen of the amide. Now what you have is a system that is activated for nucleophilic attack by a nucleophile. That's the generic mechanism. There is a generic mechanism where you simply phosphorylate this. Now, if this is positively charged, this is activated for nucleophilic attack, and then you lose phosphate. People have studied this over the course of years, and the mechanism for this is understood. I don't have the structures but, again, this enzyme and then the next enzyme in the pathway use the same sort of approach. The next enzyme in the pathway takes the amidine. What it's going to form is a cyclized product. This is aminoimidazole ribonucleotide. So we finally found-- Remember, I told you, you form the imidazole ring, and then you're going to put on the pyrimidine ring afterwards.

How does this happen? It looks sort of wonky. But what you can see is that this guy-- so let's just put a box around this guy-- becomes this guy. This guy is where we're doing the chemistry. That's the one we're going to attach, we're going to phosphorylate. What you have here, now, is an intramolecular attack. So, the nucleophile, instead of being ammonia, which is external, now happens intramolecularly. In the end, after you activate this, you get intramolecular chemistry. This was the site. This was the site that was activated in the

beginning.

The chemistry in these two systems is pretty much the same, and now we've got our imidazole ring, and now what we need to do is build up the rest of this system. Is everybody with me, or am I going too fast? I'm probably going too fast. Anyhow, that gives you the generic mechanism for this. I didn't draw the structures all out. The folates we've already talked about. So I'm not going to talk about that again. We're going to see the folate-requiring enzyme again later on in the pathway. Now the pathway just repeats itself. Really, I think what's most striking, this is really an ancient pathway. There are huge numbers of ATPs used in this pathway. I think, if any of you wind up thinking about cancer therapy and stuff, and whether you have de novo biosynthesis because you need a lot of purines fast, or whether you use salvage, this really requires a huge amount of energy to make this pathway actually work.

Now we have this molecule, and then the next step in this pathway. In the human system, what you do in the human system-- this-- it's not right. This enzyme, cross this off. This is a Bankovic's lie. Cross that off. It doesn't use ATP. So you need to cross that off. It just picks up CO2. If you look at this, what do you have happening here? We're going to go from here, and we're going to pick up CO2 there. CO2 actually can react really rapidly at this position. So you need CO2, and let me write this down. No ATP. I don't know why. I probably didn't look at this very carefully, but there's no ATP required for this step.

What's unusual? Do you think it's unusual to use CO2? This is called PurE. How much CO2 is there inside the cell at physiological concentrations? Think there's a lot or a little? Where have you seen CO2 used before? Remember fatty acid biosynthesis? Do you use CO2 in fatty acid biosynthesis? Anybody remember?

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: The what? Anybody know how you-- do you use CO2 directly?

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: We'll use bicarbonate? OK, why do you use bicarbonate? That's where the equilibrium is at pH 7. There is almost no CO2 unless you go down to acidic pHs, so almost no enzymes use CO2. So this is unusual. That's also true of biotin.

And, in fact, so this is the human enzyme, and it generates that product. In bacterial systems, it turns out that it does use bicarbonate and ATP, and generates a carbomate of the same

molecule. What do we know about the stability of carbonate? So, number one, why are we using bicarbonate and ATP? Have you seen that before? What does ATP due to the bicarbonate? We just saw this reaction two seconds ago. What does ATP do to bicarbonate?

AUDIENCE: Phosphorylates it.

JOANNE STUBBE: Yeah, phosphorylates it. You need to neutralize your charges. These are all magnesium ATP and you form carboxyphosphate, which has a lifetime on the order of a millisecond. That's the way biotin is made inside the cell. Almost all organisms do not use CO2, they use bicarbonate. And to activate bicarbonate, the other reason-- What's wrong with CO2? How do you hold on CO2? You think that's easy to bind in the active site? No, there's nothing to hold on to. There's no charge. It's symmetrical. So what nature does is put bicarbonate, which is charged, into the active site, and uses ATP to phosphorylate it, to form carboxyphosphate, which then reacts with the nucleophile, in this case the amino group. Yeah. Did I screw something up?

AUDIENCE: So you do need ATP?

JOANNE STUBBE: You do need ATP for the bicarbonate-dependent reaction. So, there are two different reactions. This is eucaryotes, and this is bacteria. They have two different pathways. I think this is sort of amazing, because what happens now is the bacterial enzyme then takes this, and generates this. So nobody even knew that this intermediate-- my lab discovered this a long time ago-- existed on the pathway. Why? Because its half-life is on the order of 15 seconds.

Carbomates. That's how you carry CO2 from the tissues back to the lungs. It's carried on the surface with lysines forming carbomates, and what's striking is that these enzymes-- one enzyme uses this substrate, one uses this substrate. The proteins are structurally homologous to each other. Nobody really understands that. Nature has done a shift on what normally happens in the eukaryotic system. CO2 is added in the procaryotic system. You need bicarbonate, and you need CO2, and I think that tells you something about where these things evolved. What's the pH? And was there enough CO2 to be able to do these kinds of experiments over the evolution of these systems? I think things change.

You've now produced this molecule, which is called CAIR. So, that's carboxyaminoimidazole ribonucleotide. Then the next step in the pathway. Now, we only need this and another carbon to complete the pyrimidine ring. It turns out aspartic acid-- which is also a major player in

pyrimidine biosynthesis-- this nitrogen is going to come from aspartic acid. What are we going to do? We need to activate this carboxylate to attach the amino group of aspartic acid. How do we do that? With ATP. We phosphorylate it, and then we have nucleophilic attack.

I'm going to go up onto the board up there, so you can still see what's happening. This next reaction. CAIR now reacts with aspartic acid. And we need, again, ATP, and we go to ADP. Now what we have, aspartic acid, we form an amide linkage. R is ribose 5-phosphate. What we've done now is attached-- we're deviating, but we're going to see it near the end of the purine pathway. We use this strategy again. Almost all the time, if you have to guess at this, the source of ammonia or nitrogen is going to be glutamine. So if you don't know and you're seeing a new pathway, use glutamine. But here's an example where nature has used something different. She's used aspartic acid. And the ATP is, again, activating the carboxylate. So we're using the same strategy over and over and over again.

Then the next enzyme in this pathway. What are we going to do? We convert this intermediate called SAICAR. We now lose fumarate. Where have you seen fumarate before? Does everybody know what fumarate is? That's an intermediate in the TCA cycle. This is an anaplerotic pathway, and you've got to feed the fumarate back in. What are we doing here? We're going to lose fumarate, which has the two carboxylates transfer the double bond. We're going to do an elimination reaction.

The next step in this pathway is catalyzed by PurB, and we'll see that nature uses the same strategy to convert IMP into AMP at the end of the pathway. Uses the same enzyme, actually. So you lose fumarate. So what we're doing now is we're going to do-- actually, the enzymes have been very well studied. We have structures of all these things. You use fumarate.

Now what we have is this guy. And this guy actually has now been found as a regulator of glycolysis. So we're linking now. You're going to see this, and I think you're going to see more of this. The only reason these guys have been found is this guy's pretty stable, so people can find it using metabolomics. But this molecule is a regulator of glycolysis, and I think the more we look, the more we're going to find basic intermediates and metabolic pathways controlling fluxes through other things. We need glycolysis to ultimately generate energy, because we need a lot of ATP to synthesize things, but the connections between all these things, I think, remains to be established.

So this is involved in regulation of glycolysis. If I'd had another couple of lectures, I would have

showed you how that fit in. And then, where are we? We're not very far away. We only need one carbon left. Where do we get the one carbon from?

AUDIENCE: Folate.

JOANNE STUBBE: Yeah, from folate. So here we have it again. Now we have N10-formyltetrahydrofolate. That's why I spent the time in the beginning. And this guy, through the same kind of a mechanism, is going to be attached to that guy. Once we have the one carbon there, then you can cyclize. You attach that. Now we're ready to cyclize and lose a molecule of water.

So, the last step is cyclization and loss of water to form inosine monophosphate. Inosine monophosphate is the end goal. That's the first time we now have it purined. So we have both the imidazole ring and the pyrimidine ring, generating this purine, which then is the branch point to form GMP and AMP. Both of these are going to involve two steps. And this tells us something about the overall regulation of the pathway. Pathways are often regulated by feedback inhibition. The M-products can come back and inhibit the first step, so things don't build up. If we come over here, If we look at PurF, This is a stop. These are inhibitors-- our AMP and GMP.

We're going to see, in this pathway, AMP inhibits its own biosynthesis, and we're also going to see GMP inhibits its own biosynthesis. So what you see is, ultimately, we want to control the relative ratios of purines and pyrimidines, which we're not going to get to, and these are examples of simple allosteric effectors. They bind outside the active site and shut things down. And we actually understand a lot about how that works, we just don't-- we're not going to have time to discuss that.

So what we've gotten in to through all of this is inosine monophosphate. If you look at the next step in this path. If we go back here, here is IMP, and we want to go to AMP, and we want to go to GMP. If we look at AMP, what do we see? Have you seen this before? We're attaching aspartic acid. Where have we just seen that? We've just seen aspartic acid attachment. And what's interesting about this is, instead of using ATP, it's using GDP. Is that an accident? I don't know. GTP is regulating the flux to form AMP. So again, AMP, ATP, GTP, you've seen this over and over again over the course of the semester. You saw, with translation, it was all GTP. In other cases, you saw, with folding, with the proteasome, it's all ATP.

You've got to control all of these ratios. Here is a place where the ratios are controlled. So how does this happen? What are we going to do with the GTP in that molecule? We want to go

from here to here. This carbonyl is replaced with the nitrogen of aspartic acid. What are we going to do to that oxygen?

AUDIENCE: Phosphorylate it.

JOANNE STUBBE: Phosphorylate it. And that's done by GTP rather than ATP. So what you do is you phosphorylate through the mechanism that we just went through, that I wrote over here somewhere. Where did I write it? All right. I can't see where I wrote it, but it's in your notes. You then have your amino group of aspartic acid, displaces this, and then what happens in the last step? This is exactly what we saw over here. We're kicking out fumarate. So this is the same enzyme. So PurB also happens here. So it's kicking out fumarate.

Now, what about this pathway? This pathway is of great interest, because it's a major target-- when you have a transplant, to prevent rejection-- of mycophenolic acids. There are many compounds that inhibit this step in the pathway, and it's widely used for organ transplant, subsequent to the transplant. This is called IMP dehydrogenase. How do you get from here to here? This is not so trivial. What you see, and this is the unusual thing about this, hopefully now you could actually think about this, but we're adding an oxygen here. So, somehow, we have to add water, and then we're using NAD and ADP, so we're going to have to do an oxidation and NAD gets reduced.

If you look at this, what happens is this molecule is activated for nucleophilic attack at this position to add an OH here. So what you generate is-- then this guy needs to get oxidized by NAD. That's an unusual step. You should go back and you should think about that. It took people quite a while to figure this out. What about the last step? How does this work? Where have we seen this before? Glutamine. What we're doing is converting this oxygen to an amino group. What's doing that? I told you there were five glutamine-requiring enzymes in the pathway. This is one of them.

What do we need to do to this oxygen to make it into a good leaving group? We need to phosphorylate it. Use ATP to phosphorylate this, and then glutamine supplies the ammonia, and that's how you get GMP. As an exercise, you should go back and think about these interconversions. If you have trouble, you can come back and talk to me.

I put on the Stellar site a new version of a chapter on purines from a book by Appling that has come out last year. Within this section, it's by far and away much better than any of the others. So those of you who want to look at the chemistry of this, they've written this all out in detail.

So you can pull it out and flip to that page. You don't have to read the whole chapter. You can flip to the page where they describe all of these things.

So that's the purine pathway. My goal was to try to show you that everything has made from ribose 5-phosphate as a scaffold. You build up the imidazole, you build up the purine, and you use three types of reactions that are used over and over and over again in metabolism. One of the reasons I picked this topic, besides the fact that I like deoxynucleotides, which I never get to talk about, is the discovery of what we talked about in recitation 13, this purinosome.

What's the purinosome? You all know what it is, And we talked about some of the experiments, but the idea is that you have proteins from all over the place that organize transiently. So you have transient protein-protein interaction that arise to the occasion. There's going to be some signaling mechanisms that they know they're depleted in purines. That's the model. They come together, they do their thing. Why would you want to do this? The choice that everybody has looked at has been the purine pathway for this idea of multi enzyme complexes that form transiently, and I've asked you this question recitation, why would you want to do this?

One reason you might want to do this everybody agrees on, and that's because if you have unstable intermediates, and these intermediates go into solution, they can degrade. So that would be a waste. Is that true? We don't know. But one reason would be to protect unstable intermediates. A second reason that you might want to do this is if you have a long metabolic pathway-- this is tensed up, it's a long pathway-- oftentimes, in the middle, you can have branch points to other pathways. Say you want your intermediate to go this way and not that way. If you have this organized, you can control where it goes.

If you have a pathway, and you have some intermediate X, and it can go another way, so this would be a branch point, you can prevent formation going into another pathway. And in the purine pathway, this feeds into histidine metabolism and thiamin biosynthesis, and tryptophan biosynthesis. So, there are intermediates in this pathway, and when you start looking at metabolism, you find these connections all the time. We know a lot of these connections. I don't have time to go through them, but that would be another reason that you would like to be able to do that.

The reason Bankovic got into this, and that's whose work you've been reading, is he was interested in the question of whether N10-formyltetrahydrofolate-- remember we talked about

all the interconversions-- whether all of those intermediates were sequestered. That's why he got into it. And what is the answer? He was interested in this question of tetrahydrofolate metabolism-- central to both purine and pyrimidine metabolism.

And what do we know about that? In the control experiments in the paper you needed to read, what did he use as a control? He used-- remember we talked about this trifunctional protein that has three activities? It puts on the formate, it does a cyclohydrolase, it does a dehydratase. So if you go back and you look up the enzyme in his notes, this is not in purinosomes. And that's one of the first experiments he did. It's not there. So why isn't it there? I don't know. And maybe that means that we should be thinking about these things in other ways.

In the last minute or so. So, that summarizes the key thing. Unstable intermediates and multiple pathways, and sequestration. I think there's no debate about that. If you have things sequestered, can you increase fluxes through pathways? A lot of bioengineers say you can, other people say you can't. This, to me, becomes really important to metabolic engineering. If you read metabolic engineering papers, people will take a polymer, and they'll stick all the enzymes in the pathway onto a polymer. Why? Because they think it's important to have these things in multi-enzyme complexes, where you increase the effect of molarity. That's something else we've talked about extensively over the course of the semester.

Methods used to study this. OK, we've talked about that in recitation 13. We talked about what the issues are. In all cases, he used the enzyme fused to a green fluorescent protein. You could have problems with aggregation. You could have problems with altered activity. We talked about all of that last time. Looking at these-- punctate staining, if you look at the punctate staining with one protein and another, they're widely different. The shapes of the stains are widely different. Azaserine and hypoxanthine-- Azaserine we just talked about. Hypoxanthine-- hopefully, you now remember that that-- IMP, hypoxanthine, with PRPP, this is salvage.

You should now be able to, thinking about this, go back and read that experiment he did. That experiment makes no sense to me. That was an experiment he did because he made a prediction, knowing how all these things fit together, and it didn't do what he predicted. So then he made up something else. These are the kinds of things you need to think about when you're trying to test a model like this. It's a very appealing model, but it's also a very controversial model.

I'm sort of at the end of my time, so I think I'm going to go to the end. We've looked at all of these-- punctate staining with no purines, when we add purines, we lose it. And I just want to go to a paper that was recently published. This is probably hard to see, but this just shows this is an ongoing area of research. The latest is, now, instead of looking at this fluorescent stuff where a lot of you commented, you really can't see the green overlapping with the red to form yellow. The pictures were terrible, and if you go back and you look up there, I can't see it either. Fluorescence changes, and red and green on top of each other showing yellow showing they're sort of in the same general area are often hard to see.

So now, they've turned to super-resolution, and if you look at when you turn off the lights, this is mitochondria, and these little purple things are the putative purinosome using green attached fluorescent proteins. And what you can see is there-- and again, you need to look at the statistics of all of this-- they appear to be associated with the mitochondria. Does that make sense? I don't know. That's where you need purines to make all of your ATP. Anyhow, it's linked to signaling pathways, and they do that in this paper.

But again, to me, this is just another example. I don't think they expected to find this. And they found that, and so now we have more complex systems to really try to understand why these things-- do they sequester, number one, and if they do sequester, what is the advantage to biology?

So, we end here, and the bottom line is, when you think about all the data, it's a moving target. You can't prove something. If you're a mechanism person, you can't prove a mechanism. It keeps changing. That's the way life is. So you have a model. You make it as simple as possible, you get some data, you find something that doesn't agree with your hypothesis. You've got to change it. That's why science is so much fun. That's the end of purines, and I'm sorry I didn't get to tell you about ribonucleotide reductases. It's much more chemically complex than anything you saw in purines, so I am sure you are delighted that you didn't have to look at all the radicals. So we'll see you on, I guess, Tuesday.