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JOANNE STUBBE: We talked last time about kinetics, steady-state kinetics, pre-steady-state kinetics, how you design the experiments, what kinds of information you can get out of each experimental design. And we introduced all of that material. And today, what I want to do is come back to the model. You saw it at the very beginning, and you've seen it in a lecture. And specifically, where did this model come from? That's what we're going to focus on.

OK, and so in order to be able to understand this model, you have to design assays. And you're going to see over and over again over the course of the semester figuring out how to design an assay, in this case, isn't so hard, but in many cases is really tough. And that's the key to being able to get kinetic information is designing the assay.

So if you look here, today, we're going to be looking at GTP is hydrolyzed. So you need to think about, as a chemist, how you could study that reaction. How would you look at starting material? How would you look at product as a function of time, which is what we were talking about last time? And we're going to talk about that first.

We're going to talk about use of radioisotopes first. And we've already been talking about radioisotopes in class the last couple of lectures. So we decided to focus most of our energy now on radioisotopes.

And then the second kind of probe you're going to see is a fluorescent probe. We're going to use fluorescent probes over and over again. And the details of the fluorescent probes and how they work isn't going to come in until the last recitation, which is recitation 13.

So from the point of view of thinking about Rodnina's paper, what you need to think about is, if you have a probe, and you stick it in a different environment, it changes. And you can watch it change, OK, without looking at the details. But that's something you do need to think about, but we're not going to talk about that.

OK, so we have a way of monitoring potentially GTPase. And we'll talk about that today. What

other reaction can we monitor in here? We can monitor formation of the polypeptide chain. And so that's the other thing.

And both of these chemical transformations use radioactivity. OK, so that's where we're going to focus on it initially. And then hopefully-- how many of you went back and reread the paper for this week from last week? Did any of you go back and reread it?

OK, so I think it's good. I just think, you know, every time I read a paper-- I read a paper. Sometimes, I've read it 10, 15 times over the course of my career. And as I learn more and think about things differently, I keep seeing new things. And this paper is just packed full of information. So I could say you could read it another 10 times, and you'd still keep learning stuff out of reading it.

And in the very beginning, that's what we're trying to teach you to do. What do you look at in the paper to learn how to critically evaluate what's being presented in the model, which is maybe what you're going to build your research program on? Somebody else's data, is it correct? Is it not correct?

OK, so we're going to use radioactivity. I'm going to start there. And then to look at these first few steps, which are binding steps, that's where we're going to look at the fluorescent probe.

And there were three different kinds of experiments that were described in this paper-- looking at the rates of the reactions as a function of the concentration of the ribosome-- you need to think about why they looked at a concentration dependence-- measuring fluorescence changes, and then they used non-hydrolyzable GTP analog. Why did they use that? Do you remember what the non-hydrolyzable GDP analog was? So where's the n?

AUDIENCE: It's between beta and gamma.

JOANNE STUBBE: So it's non-hydrolyzable. It is hydrolyzable, but not under the experimental conditions. So what does it do? Why would you want to use something like that to get information about the first few steps?

AUDIENCE: It's along the reaction continuum.

JOANNE STUBBE: Yeah, so you don't let the reaction continue. So what that does, if it's working correctly, is it puts a block here. And then you can potentially monitor what's going on here. And from the data that you looked at, it's not really so clear what was going on there unless you went back

and read the preceding paper. So there had been a decade worth of experiments on this system before this paper came out summarizing the conclusions about what they are thinking about fidelity.

OK, so what we're going to do is talk about radioactivity. And our objective is simply-- and we'll come back to this at the very end-- is to use all this experimental data, the concentration dependence, the radioactive isotope experiments, the stop flow fluorescence experiments, and try to come up with a model that can explain all of the data.

OK, so you make some measurement. What you're measuring is some k apparent. And that's usually a first-order rate constant because it's happening on the enzyme. OK, so you measure these numbers.

Well, what do they mean? You don't know what they mean. And why don't you know what they mean? Because the kinetic mechanism is so complicated. You saw that with the steady-state analysis of km and kcat last time.

So in the end, though, if you come up with a model, and it can explain all the data because you've done many, many experiments, it can be quite informative about the question we're focused on is specificity. How do you distinguish between phenylalanine and leucine and proofreading? How do you decide whether you're going to form the right peptide bond or the incorrectly charged tRNA is going to dissociate?

OK, so that's what you want to come out with. You want to look at the ratio of these rate constants and the ratio k3 to k minus 2. And when you look at the experimental data, which we'll look at the end today, it should make sense to you in terms of this model.

OK, but let's put it this way. In most cases, you don't come out with a unique model. It's a working hypothesis that people for the next 15 years, if it's an interesting problem, will take pot shots at to try to understand in more detail what's really going on.

OK, so what I want to do is talk about two methods, but the focus probably won't get very far in terms of the second one. But today, we're going to look at radioisotopes and how you use that to do the assay for GDP hydrolysis and peptide bond formation.

OK, so what is an isotope? OK, so how many of you guys have actually worked with radioisotopes? Any of you? No, OK, so you know, maybe they don't use this anymore. Biochemists for the decades have used isotopes. Every paper I read has isotopes in it. But you

know, I'm old school. So maybe people don't use it.

But I think the power of it is its sensitivity. I'm going to show you that today. And the other power of it is that you have no perturbation of your system and there are almost no probes like that. You're sticking on green fluorescent protein.

Well, what does it do to the whole rest of the protein? You have to perturb to see, but radioisotopes have minimal perturbation. So it's still a very important probe, but it probably depends on what kinds of questions you're focused on.

So what is an isotope? So an isotope is atoms with the same number of protons and a different number of neutrons. That's called the mass number. So what you have here for carbon, which is one of the common isotopes you guys will be using if you do any kind of biochemistry, we have C-12, C-13, and C-14. OK, and so this is the atomic number, which is the number of protons.

OK, so the only difference between these guys is a neutron or two neutrons. OK, so there's minimal difference. And so what are the isotopes that you see used in biology? So we've already seen many of these in this paper, but we've also talked about some of them in class today and in the preceding class.

So we're going to be using over the course of the semester isotopes of hydrogen. Why? Because if you look at your metabolic pathways, you're always cleaving carbon-hydrogen bonds. OK, so this isotope becomes incredibly important. C-12, C-13, anybody know where you use C-13?

AUDIENCE: In NMR.

JOANNE STUBBE: NMR, so if you're working for Mei Hong, you might be doing isotopic labeling using C-13. If you're doing any kind of metabolic label chasing, you're going to see the radioisotope is, which is what we're talking about, is C-14. Working

So you see often, all the time, you see nitrogen and oxygen. And oxygen has three isotopes. Nitrogen has two. None of them are radioactive. OK, so you're never going to be using the methods we're describing today. But frequently, in NMR again, you might replace N-14 with an N-15.

And today, we will see that we're using isotopes of phosphorus. What about phosphorus-31?

Where do you see that? Have you thought about this? Maybe you have, and maybe you haven't.

Phosphorus-31 versus phosphorus-32, what's the normal abundance isotope of phosphorus? 31, so phosphorus-31 has a nuclear spin of a 1/2. So you frequently use that as well in NMR. And P-32 is used-- it's radioactive and is used in today's experiments.

OK, so this is something that in the back of your mind you should think about. What are stable versus unstable isotopes? And what we're talking about today is unstable isotopes.

So what I want to do is we're not going to go into this in a lot of detail, but I want to describe the things I think you need to think about if you're ever going to use radioactivity and how you make measurements, quantitative measurements. And so we're going to be looking at a radioisotope. And what do we know about radioisotopes?

They're unstable. OK, and depending on which atoms they are, they have different stabilities. And they decay spontaneously into some new configuration. They have a nuclear decay spontaneously into a new state.

And during this process, during this decay, they emit ionizing radiation. They emit energy. So during this process-- so this is the whole thing you need to remember. They emit energy.

And the energy is in the form of ionizing radiation. It could be alpha, beta, or gamma radiation. And so what we're going to be looking for is trying to detect that energy that's actually released.

OK, so before I go on, we've already seen all of these isotopes used already in class, even though we've only gone through seven lectures. And when we look at the LDL receptor and cholesterol homeostasis, there aren't very many LDL receptors. You something highly sensitive, which is something that you need to think about. And I-125 which is a gamma emitter, is what you end up using. We'll come back to that in recitation, I don't know, 8 I think it is.

OK, so they're unstable. And they spontaneously decay into a new configuration. And they release energy. And what we want to do is detect the energy. The ones that most of you will be focused on, if you use radioactivity in your experiments, will probably be all beta emitters. And all of these guys over here are beta emitters. And you've already seen all of these

radioisotopes.

OK, so what do we know about these isotopes? There's two things you need to think about. So this is the properties. And one of them is the energy of the beta particle or gamma particle released. And if you look over here, what do you see?

Tritium has 18.6 with this kind of unit. The unit might not mean very much to you. All I want you to do is look at the relative energies. Versus phosphorus, 1,710, so it has much more energy released. And what does that mean?

If you've never worked with radioactivity, you might not-- a lot of chemists are petrified of radioactivity. I mean you could eat most tritium and C-14. Don't tell anybody I said that.

But you could eat almost all tritium or C-14-labeled molecules you end up buying. They don't really do anything to you because the energy is low. And if you wear plastic gloves or something, that protects you from any kind of energy released.

P-32, on the other hand, which isn't used as frequently, does anybody know where that used to be use all the time? I've used tons of P-32 in my lifetime. Where do you think that was used initially?

AUDIENCE: With DNA.

JOANNE STUBBE: In what?

AUDIENCE: DNA.

JOANNE STUBBE: DNA sequencing, yeah. So DNA sequencing, which you guys don't do, you send it out to have somebody do it for you. We used to run these huge gels.

And we used to have to run many, many sequencing gels to sequence something that was 500 base pairs long. And P-32 was the method of detection. So what you do there is it's still not that bad, but you have to have a safety shield.

So if any of you use radioactivity at MIT, they have radiation safety, and you go. Even if there's somebody else in the lab using it, and you're not, you should go just read the handouts that they give you to be aware of what's going on with radioactivity. I would say the biggest issue is that, if somebody spills it and doesn't clean it up, then it can contaminate everybody's experiments in the lab. That I would say is one of the biggest issues with radioactivity.

OK, so iodine, again, is a gamma emitter. So that's in a category by itself. So the other thing that I think people don't really have very much feeling for is the half-life of decay.

And if you look at that, look at how many years for your C-14 to decay by 50% from whatever the number is, forever. You don't have to worry about it. You can sit it in your-- you can leave it in your refrigerator, and it's good for your lifetime anyhow.

On the other hand, P-32, for example, has a half-life of 14 days. So what does that mean? It's spontaneously decaying continuously. And if you have it for 14 days, you start out with some number. We'll define what that number is. And then 14 days later, you only have half as much.

OK, so you need to know something about the half-life. And the only one you need to ever think about is P-32, which they needed to think about in the experiments that are described in the Rodnina paper. So you have the energy released, and the energies are distinct. And so the question then is what we really want to do is think about quantitation. So that's going to be the key thing is we want to be able to quantitate radioactivity.

And to do that, we need a method of detection. And there are a number of methods of detection. The one that-- I guess, again, I'm not sure. I think I'm the only one in the chemistry department that has a way of detecting radioactivity using an instrument called a scintillation counter. So this is sort of a very oversimplified view of what's going on in your scintillation counter.

And so people come from all-- actually, they come from lots of places on campus to use it. So again, I think that's common. I don't know how many people are using radioactivity.

But you have radiolabeled molecule here. It would be leucine. And you have tritiated leucine. OK, or it would be P-32-labelled GTP. Those are the two molecules that are used in these experiments.

You put it into a little vessel with some kind of fluid. And the fluid that you use, whether it's organic, water, aqueous, or mixtures, depends on the molecules you're dealing with. OK, and the energy gets transferred in some way to the solvent in your solution.

And then you put in a small molecule called the scintillant, which can remove the energy from the solvent and absorb that energy in some way. And again, it depends on-- the standard one we use in my lab is POPOP. You can look up scintillants in Google, and you can find out what

the structures of these things are.

And then these things decay. And when they decay, the energy is related to the detection method using a photomultiplier tube. So it gives you a quantitative measure of how much radioactivity you have over here and how much you get out on this side.

Now, if you look at this process, it's complicated because you have energy transfer. So what can happen during this energy transfer depending on the energy of your radioisotope? Anybody got any ideas? What might you have to worry about?

AUDIENCE: You're looking at efficient transfer.

JOANNE STUBBE: Yeah, so the efficiency of the transfer. And if you have something in a solution, often, you're doing crude cell extracts. OK, so you have a lot of things in there that can also absorb the energy. So at any stage along the way, you can get quenching.

OK, and if you get quenching, that reduces the amount you detect over here. OK, so that's going to throw your numbers off. So where is quenching a problem? Quenching is a problem-- we just looked at all these energies. OK, tritium has the lowest energy. OK, P-32 has a much higher energy.

So if you look at it, and you have to figure this out for every system you work on. I've worked on tritium-labeled molecules where you couldn't be quenched by 90%. So if you have some measure-- we'll call it decompositions per minute, 10,000-- if it's quenched by 90%, you've lost a lot of your sensitivity. So you have to figure out a way to determine whether you'll get quenching or not. Otherwise, your numbers are completely off.

So why do you want to quantitate your radioactivity? Where would you be using radioactivity? And why would this quenching make a difference? Yeah?

AUDIENCE: This is just a question.

JOANNE STUBBE: Yeah, sure.

AUDIENCE: What's kind of like the nature of the solvent to the fluorescence involved. Is that like a [INAUDIBLE] kind of idea?

JOANNE STUBBE: So it's just some kind of energy transfer. Yeah, so I mean it depends on what the molecules are. An it depends on what the solvent is.

AUDIENCE: OK.

JOANNE STUBBE: OK, so every single one of these systems, you need to go in and look at the details of what's going on. And so when you do this, people have worked out these conditions so that when you're measuring-- and so this is an important question you're asking. How do you know that what you're measuring really is related to what's way over here? So that's the absolutely the right question to ask.

And so when you start, for example, the first thing you do is every scintillation counter comes with a standard. OK, and so the instrument is calibrated. And if you care about radioactivity, you have somebody come in, and they calibrate the instrument twice a year. OK, so all of this stuff is really important.

And the question of sensitivity is important. We're going to see what you're measuring is something called decompositions per minute. OK, that's the readout you get from the instrument. And so you might be getting 100,000 of these things.

But in fact, you might be getting five. OK, is five real? Five can be real if you count it so you get a statistical distribution to make sure the five is real, that it's not five plus or minus five.

OK, so radioactivity is incredibly sensitive. And you can extend the sensitivity by just counting your material in this instrument for a very long period of time.

OK, so where would you want-- where have you already seen that you would use radioisotopes? What did you see today in class, for example, in ribo-x? You looked at an experiment with ribo-x today in class.

AUDIENCE: Oh, the cysteine incorporation.

JOANNE STUBBE: Yeah, with cysteine incorporation. What were you looking at?

AUDIENCE: It limited the radioactive system.

JOANNE STUBBE: Right, but what we are using to look at this? So we're talking about the detection method. So I'm going to describe another detection method. Would you be looking at this by scintillation counting? No, so you need another method.

AUDIENCE: So like one of those phosphorimagers.

JOANNE STUBBE: Right, so I'm going to show you. That's the next thing. So what you could have, for example, is a TLC plate. Or you could have-- they were using a gel, an agarose gel probably. So you need a way of detect the radioactivity that's going to be distinct from scintillation counters where you use little vials and scintillation fluid. And you have a completely different method of detection.

And these methods keep changing. And so I don't update them anymore. I'm not sure what the current technology is. It's all secret anyhow. So they tell you sort of something about what it is, but they don't tell you any of the details because it's all proprietary.

OK, so in that case, what were we looking at? We were just looking for incorporation. We were doing some labeling experiment in the cell. OK, so we were chasing a label.

So that you're going to see a lot. That's how all the metabolic pathways were figured out. The advent of C-14 as an isotopic label revolutionized our understanding of glycolysis, fatty acid biosynthesis, et cetera.

And today, what are we using radioisotopes today to do? We're using it to do what? We're looking at GTP. We want to look at GTP going to GDP plus Pi. OK, so what are we using this for? To get information for our model.

What do we do as a function of time? Why do we want to use gamma P-32-labeled ATP? And how do we use this in analysis?

Did you even know that we're using gamma P-32-labeled GDP? How many knew that? Anybody read that in the methods section? You, over there, did you read that in the methods section? What's your name?

AUDIENCE: Mathis.

JOANNE STUBBE: Matt?

AUDIENCE: Mathis.

JOANNE STUBBE: Matt, did you read that in the methods section or not?

AUDIENCE: No.

JOANNE STUBBE: No, did anybody read it in the methods section? So I mean that's what-- again, this is what this recitation is all about is looking at the details of what's going on. And I think when you first start

doing something like you don't know what details to look for. Some of you might have read it, but it didn't mean anything. So it went in one ear and out the other. Yeah?

AUDIENCE: Wouldn't you have to use gamma labeled GTP though? I mean the hydrolysis gives you GDP and phosphate. So that's the only-- I mean, if you labeled another one, it doesn't give you as much information.

JOANNE STUBBE: OK, so if you labeled-- say you labeled the base. So let's just call this base. Say you put a tritium in the base, OK, versus-- hopefully, you all know this, but this is the gamma position versus a label here. Why are you putting the label here? What's going on in this reaction?

Actually, this was interesting because this is the second recitation where I don't think anybody understood what was going on in this reaction, which is rather disturbing. What's going on in this reaction? So we're going GTP. So this is G. So you have a nucleoside and three phosphates, TP. And what are you producing out the other side? GDP. So what's happening during this reaction? Yeah?

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: Yeah, so you're hydrolyzing it. So in some way, that's what all of these GTPases are about. You're going to see these GTPases not only in translation. You're going to see it in three of the sections that I talk about. GTPases are everywhere.

OK, so what you're looking at is then some way you have hydrolysis of the gamma phosphate. OK, so why are you labeling the gamma phosphate? You could have labeled actually the alpha or the beta.

AUDIENCE: You wouldn't be watching the reacting.

JOANNE STUBBE: Yeah, you want to watch your reaction. So if you have an isotope here, which we're going to watch it using some method, scintillation counting or phosphorimaging, and where does the label end up? The label ends up here.

OK, well, if you put the label in alpha or beta, could you follow the reaction? OK, well, you're shaking your head no. Why couldn't you follow the reaction?

AUDIENCE: Because it would stick in the GDP [INAUDIBLE]--

JOANNE STUBBE: So it would be in GDP.

AUDIENCE: --there's no GTP in GDP.

JOANNE STUBBE: Yeah, is there a difference chemically between GDP and GTP? Again, this is what I'm finding. You need to think about the structures of everything you're working with. We're chemists. OK, what is the difference between the diphosphate and the triphosphate?

AUDIENCE: It's harder to hydrolyze the next phosphate off.

JOANNE STUBBE: Well, it's not. You know, all of these things-- without an enzyme, all of these things are hard to hydrolyze. Why? Because you've got negative charges all over the place, and a nucleophile can't get into the active site. So they're all hard to hydrolyze. So that's not-- you have to think about that, but that's not what I'm looking for. Yeah?

AUDIENCE: So if you run a gel or something, they should come out-- GDP and GTP are going to come out in the same-ish area, whereas, obviously, phosphate--

JOANNE STUBBE: OK, so that's what you need to think about. But what can you take advantage of as a chemist where they don't come out in the same-ish area?

AUDIENCE: I mean, if you label them, the gamma phosphate, then the label won't come out nearly anywhere.

JOANNE STUBBE: So that's absolutely true. But what is it about this molecule? Because I've been sloppy. What is it about this molecule that allows the distinction between your starting materials and products? This is what developing an assay is all about. How are you going to monitor this reaction?

So in this paper, one of the graphs looked at Pi production. We're going to look at this if we get this far. OK, so how would you distinguish between these things as a chemist? You have no idea. You, you haven't any idea, not good. OK, what about you?

This isn't a hard question. Look at the structures. And as a chemist, how would you distinguish your starting material from your products? That's the question.

And that is the question in any assay you have to develop. That's what you've got to figure out. You've got to figure out a way to distinguish the starting materials from the product.

Now, if we have a base here, and if this is G, we have a base here. What do we know about guanine? What's its absorption look like? What's its absorption spectrum look like?

AUDIENCE: 210 [INAUDIBLE].

JOANNE STUBBE: How much?

AUDIENCE: Isn't it like 210 nanometers.

JOANNE STUBBE: I can't hear you. You need to-- don't mumble. Look at me in the face and tell me. You know, don't be shy. I mean, we all ask questions. [INAUDIBLE]. We're here to learn. Right? Yeah?

AUDIENCE: It absorbs in the UV. I think it's 210 nanometers.

JOANNE STUBBE: OK, so it's not 210. So you guys need to go think about amino acids and nucleic acid. It absorbs at 260. OK, so I mean, potentially, you could sit at this absorption at 260.

But what does GTP look like? GDP look like? It has the same base. So you're not going to see any change. So that's useless because you need to be able to monitor a change during the reaction.

OK, so what else about this molecule will easily let you, as a chemist, determine substrate from product?

AUDIENCE: The charge.

JOANNE STUBBE: Yeah, the charge, yes.

AUDIENCE: Just do anything with the charge.

JOANNE STUBBE: So here we have all of these negative-- every oxygen is negatively charged. Here we only have two phosphates. Every oxygen is negatively charged. Phosphate-- all right, let me ask this question. We'll see how much we need to be thinking about here.

So we have-- what is the charged state of phosphate? Can anybody tell me?

AUDIENCE: Minus 3.

JOANNE STUBBE: Pardon me?

AUDIENCE: Minus 3. It depends on the pH of your solution.

JOANNE STUBBE: Yeah, well, we're at neutral pH. So you look at all the buffer. You know what the buffers are. They've described the buffer in their reaction. So you're at neutral pH. What is the charge?

AUDIENCE: Minus 2.

JOANNE STUBBE: Yeah, so it's the pKa of the first proton loss is at 1.6. And the pKa of the second proton loss is about 6.8. So you'll have a mixture between 1 and 2. So this is incredibly different from this.

And that makes it-- how do you separate things? By an anion exchange column, which separates things based on charge, some kind of a TLC system, which can separate things based on charge. And so that's what you have to do in your overall assay.

OK, so the second place where you're going to use radioactivity is an assay. OK, and in the paper you read, not only did they use it for GTP, they had to use it to monitor peptide bond formation. Can anybody tell me how they did that?

So what are we looking at if we go back to the original? What's the product of the reaction of the EF-Tu reaction with the ribosome? What's the product you get out?

AUDIENCE: [INAUDIBLE] on EF-Tu and also label the hydrogen on leucine.

JOANNE STUBBE: OK, so you're labeling the hydrogen on leucine. OK, but then what are you looking at in your assay? We're developing an assay. Here we're developing an assay where GTP is going to GDP plus Pi. What are we looking at in the case of the leucine in this experiment?

AUDIENCE: The leucine is incorporated into the peptide. And you have the [INAUDIBLE].

JOANNE STUBBE: OK, so but where is the dipeptide? So that's correct, yeah.

AUDIENCE: It will be in a P [INAUDIBLE] on the ribosome.

JOANNE STUBBE: Yeah, but what's it attached to? Is it a dipeptide?

AUDIENCE: Yeah, it's attached to the less phenylalanine.

JOANNE STUBBE: Yeah, and what is that attached to?

AUDIENCE: Another tRNA.

JOANNE STUBBE: What's the phenylalanine attached to? If you look over here, what is everything attached to?

AUDIENCE: Another tRNA.

JOANNE STUBBE: It's attached to a tRNA. So could you separate a tRNA with one versus two amino acids chemically? Is that easy?

AUDIENCE: No.

JOANNE STUBBE: Now you have charges. Right? You have huge numbers of charges on your RNA. But they're the same on all the tRNAs. So you have one amino acid, which has a carboxylate end and a second amino acid, which has the same charge. Do you think that's going to be easy to separate? No. So does anybody know what they did to make this assay work?

AUDIENCE: Put the label on leucine so the leucine is incorporated. Then you're still different [INAUDIBLE]. You can have basic number. Then after the conversion, you have a signal.

JOANNE STUBBE: OK, so after conversion, you have a signal. But then the question is how do you detect this. So you have-- I mean, I guess what they could have done-- so we started out with a leucine that's labeled. And so what you're saying is that you have a way of detecting your leucine on the tRNA.

So this is all attached through an ester linkage. So this is attached to the tRNA. So what you would be after is separating an amino acid from a tRNA. So that's possible. You could potentially do that.

But what do you think about the ester linkage? This is all the thought process that goes into an assay and making an assay robust. Do you think that ester linkage is stable?

You're going to have to chromatograph it someway to separate your starting material from product. So the answer is it's not very stable. And if you don't know, you've got to figure that out.

So what they do is they quench the reaction with hydroxide. OK, and why did they quench the reaction with hydroxide? So this is a rapid chemical quench like we talked about last time. Why did they do that?

AUDIENCE: To hydrolyze the ester.

JOANNE STUBBE: Exactly, so then what do you have? You have, you know, your dipeptide here. Or you could hydrolyze before. And then you would have no label at all. And so then you can monitor dipeptide formation.

So if you looked at the details of the graph that they presented, they weren't looking at tRNA charged with a dipeptide. They were looking at the peptide. And so that should have been a clue. Immediately, you go back to understand what's going on in the assay.

So you have assays. This is pretty important. And where's another place where you might want to use radio label, where you need a sensitive assay? We're going to see radioactivity is incredibly sensitive. I'm not getting very far.

But what other kind of an experiment might you think about if you have some kind of a mammalian cell, and you have receptors on the cell, for example? And you don't have very many receptors on the cell. You have, you know, sub-nanomolar number of receptors. Where else might you want to use radioactivity? And we're going to see this in the cholesterol section again. Anybody got any idea?

So you have some receptor on a cell. And you want to count the number of receptors. We need a quantitative way of looking at that.

AUDIENCE: We need to measure uptake.

JOANNE STUBBE: So uptake is another place. You absolutely would want to use it to measure uptake. It's frequently used also to measure binding.

OK, so you have to figure out a way to prevent-- on the cell, you can prevent uptake by just cooling down the lipids. And then you're measuring binding. OK, and that's exactly what they did in the LDL where they count the number of LDL receptors.

So the other place where you're going to see this used over and over again is some kind of binding assay. And there are many ways to measure binding. You're going to have a whole recitation on this. Most of them aren't as sensitive as the radioactive methodology.

OK, so let's move on after that long digression. OK, so what you need then is a quantitative way to measure radioactivity. OK, oh, the other thing I wanted to just point out, as you pointed out before, there's another way of detecting radioactivity using a phosphorimager. And you can read about this in detail.

So what you do is you have your gel or a TLC plate. You have an image plate on top of it that somehow collects all the energy emitted from your radioactive decay. And then you quantitatively release that energy in a way that allows you to quantitate the amount of

radioactivity you have on your spot on the gel or your spot on the TLC plate.

And for example, tritium, with the lowest energy, you might have to put a plate onto your gel for a month and a half. That's how insensitive it is. You don't have enough energy to collect enough data to give you some kind of an answer. So you need to think about the energy, and you need to think about the method of detection.

Tritium is the cheapest. It's the easiest to get your hands on. s it's the least sensitive because of the low energy that's released.

OK, so the other thing that I think is amazing about the phosphorimager is, if you look at the linearity of detection, it's linear over five or six orders of magnitude, whereas, in the old days, you used to use some kind of film on top. And the film would absorb the radioactive decay and make a spot. And that was linear over a period of over one order of magnitude. So you had nonlinearity.

So that was really hard to do quantitation. So phosphorimager have revolutionized what you can do in terms of analyzing TLC or gels like Liz talked about today in class.

OK, so what we need then is a quantitative way of actually measuring radioactivity. And what is the standard for radioactivity we use? And so the quantitation and the standard is called a curie. It also could be called becquerel after the discovery of radioactivity. And there's a relationship between the two.

And what we know, the standard of radioactivity with the Curie is defined as the substance that decays at 3.7 times 10 to the 10th disintegrations per second. So one curie equals 3.7 times 10 to the 10th disintegrations per second. Or the number that you often see is 2.2 times 10 to the 12th disintegrations per minute. So this is often what you see on the bottles when you actually buy radiolabeled material.

OK, so again, what you see is you're counting. Efficiency, as I've already described, varies with the energy that's released. And you have to think about quenching. That was just repeating what I've already told you.

OK, and so then what do you do? So when you purchase radioactivity, how does it come? OK, so you guys are used to purchasing something from Sigma or Aldrich or wherever you get it. You look at it, and you can see something in the bottle. When you purchase radioactivity, you can't see anything. Why? Because there's no material, almost no material in your bottle. It's all

radioactivity.

So if you put it in a scintillation counter, you would have, you know, 10 to the ninth decompositions per minute, OK, but no material. So you can't work with it because you can't weigh it. You can't do anything with it. OK, you have-- I don't know-- a picomole of material. It depends on the material that you buy.

So the question is what do you do with this material when you get it. Well, you want to be able to use it. And in our case, how are we using it? We're going to buy GTP that's gamma P-32-labeled. To be able to use this, we need to measure something.

So what is the first thing we do? Has anybody got a guess? You can't use what you buy because what you buy is you'll get a little vial like this. And that's what you see, or you might be able to see some red material that's decomposed material actually. Yeah?

AUDIENCE: You need like a kinase that'll exchange the phosphate with the radioactive phosphate.

JOANNE STUBBE: No, I mean, you could do that if you wanted to convert it into something else. But we want the gamma P-32-labeled ATP. That's what we want to use in our assay. So what do you do to make this usable?

AUDIENCE: You add some buffer.

JOANNE STUBBE: Do what?

AUDIENCE: You add some buffer to the [INAUDIBLE].

JOANNE STUBBE: You add some buffer. OK, does that change the amount of material? No, so we probably do have some buffer, OK, because we want to be able to transfer it into something so we can do our assays. So go ahead.

AUDIENCE: Yeah, then we're going to transfer it when you have a specimen that you are going to take some buffer [INAUDIBLE].

JOANNE STUBBE: OK, so you can, but you have no material in there. So if you had a substrate that was 10 to the minus 12th molar in solution, would the enzyme ever turn it over? Probably not, because it could never find it. OK, so that's not going to work. So what is the-- go ahead. What would you do?

AUDIENCE: Like would it matter if like the radiolabeled phosphorus were just like a fraction of regular phosphorus? Like could you add some like unlabeled phosphorus?

JOANNE STUBBE: Exactly, and so this is the key point. The first thing you do is you take unlabeled material, and you add it into the radiolabeled material. And how much you add depends on what you're using it for.

So if you're going to use assays, and you don't need a very sensitive method, you can add much more. If you're going to look at a binding consent, you know you're pushing a lower limit of detection because you have some estimate of the number of receptors. Then you would add much less.

So what you're going to do then-- the first thing you do when you get radioactivity is you add unlabeled material. And I think this is something, if you didn't get anything out else out of today's discussion, I think most people won't get this. When you work with radioactivity, most of material, one molecule, only one molecule in 10 to the sixth to 10 to the ninth is radioactive. All the rest are non-radioactive.

OK, so this is just telling you about the sensitivity of the method. Somehow using a scintillation counter or using these phosphorimagers, you can quantitate the amount of radioactivity you have present. So when you're dealing with radio label, most of it is unlabeled.

OK, so what does that tell you then? So again, the amount of stuff is tiny. When you add cold material, what does that allow you to do? What that allows you to do is measure. And this is the key take-home message. Now you can measure the specific activity of your material.

OK, so you bought radiolabel. Let's say tritium. And then you added protonated material. And the specific activity is the amount of radioactivity per the amount of material that you have present, the number of moles of material. So it's in decompositions per minute per micromolar, decompositions per nanomole.

And again, you have to change everything to accommodate quenching effects. So what you measure from a scintillation counter is counts per minute, which is just decomposition per minute times quenching. So if there's 50%, you see half as much as you should be seeing.

So specific activity is given in counts per minute per amount, which is usually in micromoles or nanomoles. So if you know you have 1,000 counts per minute per nanomole, and you count 100 counts, how many nanomoles do you have? So you're given your specific activity. You do

an experiment.

You have 1,000 counts per minute per nanomole. And when you count this-- whoops, when you count this, you end up with 100 counts. What amount of material do you have?

AUDIENCE: 21 moles.

JOANNE STUBBE: Yeah, so that's it. So that's the quantitative relationship you need to remember to do all these assays that are actually in the paper that was described. So let me just give you two examples of this. We're already late.

But so this is tritium. OK, does anybody see anything weird with tritiated cytidine. So this was taken off of Google from Sigma. You can buy this from Sigma now. Do you think that's reasonable that we CT3 in our methyl group? So T is for tritium.

What did I just tell you about our material? How much material has got a label in it? How much--

AUDIENCE: One in 10 the the fourth.

JOANNE STUBBE: Yeah, so we don't have very much that's labeled. Say we had 100% labeled. Do you think that would be an issue? Say we had a million to-- 10 to the sixth to 10 to the ninth more tritium. What do you think that might do in terms of energy? Yeah?

AUDIENCE: You said tritium is much weaker. We're talking about phosphorus here. So that's like a huge signal, whereas--

JOANNE STUBBE: So even with tritium, OK, you still get enough energy. If you tried to put that much tritium in your molecule, within as fast as you could isolate the material, it would be completely decomposed. So there are ways to put tritium into the molecule, but the decay would completely destroy your molecule because you have so much radioactivity.

So this, which is on the web, is completely incorrect. So what you have is one molecule in 10 to the sixth that actually has tritium labeled. And how much you have, you don't know.

What you need to do is add cold material, and then you need to figure out a way to quantitate the amount of material, leucine or GTP. And then you count that amount of material. And that gives you the specific activity.

So let me just say one more thing. Those of you who have to go, I'm sorry I'm late. You can go So where do you get radiolabeled material from? Do you think this is easy? I mean you could buy leucine. I just showed you we could find that on the web. You can buy a gamma P-32-labeled GTP as well.

Most things you can't buy. OK, so this is what distinguishes a chemist from a biologist in many cases because I could make things radiolabeled decades ago. Doing a 15-step synthesis, I was able to make molecules that allowed me to study something that nobody else could study.

So the question is you need to make your label and put it in a specific position. And so what do you start with? You start with something that's easy to work with. And you try to put the label in at the very end of your synthesis.

And one of the things that you often start with is sodium borotritiride. Why would you start with sodium borotritiride? What can sodium borohydride do?

This is frequently used. We'll see this used later on. Anybody remember what sodium borohydride does? Yeah?

AUDIENCE: It's reductive.

JOANNE STUBBE: Yeah, it's a reductant. So you can reduce a ketone or an aldehyde to an alcohol. OK, so that's frequently used to put in tritium.

So what are the issues with sodium borohydride? Again, this is something that you need to think about the chemistry. What are the issues with sodium borohydride?

If you're going to put your label in, OK, I just told you. How much of your sodium borohydride is labeled? What do you have mostly in there? Do you have NaBT4?

No, so what do we know about tritium versus hydrogen? I guess this might depend on how much organic chemistry you've had. Tritium versus hydrogen, what's the difference? Two neutrons, OK, but it's huge in terms of weight, OK, because neutrons are the same weight as the protons.

So what you see is an isotope effect on the reaction. So when you use sodium borotritiride, the activity is never the same as what you got out of a bottle. You have an isotope effect.

The other thing is, if any of you have ever worked with this, and you're doing this in aqueous

solution, what does sodium borotritiride do? Anybody got any ideas? In water, at pH 7.

AUDIENCE: Proton exchange.

JOANNE STUBBE: Proton exchange.

AUDIENCE: You get hydrogen gas.

JOANNE STUBBE: You get hydrogen gas. You get hydrogen gas. The whole little flask would hit you in the face with the hydrogen coming off when you're in-- and what would you get?

You'd get a face full of tritium, tritiated hydrogen. OK, tritiated hydrogen is not so bad because it's not very soluble. So it goes into your system and gets washed out.

If you were producing tritiated water, that's bad. So that's the other place where you do this. You can get very hot labeled tritiated water. And that you have to be really careful of because, if you breathe that in, it gets mixed with all the unlabeled materials. And it takes forever to get rid of it.

So I think we're not even going to get to-- I'd let you go through all of this. But what I want you to do now is go back and think about what this data means. At least you now know what the assays are.

And think about the axes. And think about, you know, cognate versus near-cognate. Why do we see a lag here? What happens at 100%?

You're using up all the GTP. What does that mean? That's what I want you to think about.

If you come over here, and you're looking at dipeptide, not tRNA. We're looking at a dipeptide. You need to look at the axes. They're completely different. One is micromole. One is nanomole.

So we're trying to get you to actually look at the primary data, which you may or may not-- how many saw this difference when you read the paper? OK, so to me, this is what we're trying to get you to do on the first test. I can tell you a lot of people have trouble looking at this. That's what we're trying to get you to do. That's why we're going through this in so much detail.

And then it becomes second nature. You just start reading it. You look at the details. And you make a judgment. If you don't understand what's going on, you go look it up, or you go talk to

somebody about what the issues are with the method.

Here again, the lag phases are not all that different. But here, if you take the differences in amounts into account, you're only getting 1%, 1% to 2% the amount of leucine incorporated into the peptide as you would with two phenylalanines. And that's because it's a near-cognate.

And what's happening? You know, you're having discrimination between peptide bond formation and dissociation. So that's the proofreading part of the overall mechanism.

So I think thinking about these two slides really tells you quite a bit about whether you believe the model that Rodnina-- whether the model is reasonable given the data you actually see. All right, I'm sorry. I'm way over. So I'm going to stop here.