**JOANNE STUBBE:** Recitation 2 and recitation 3 are on the same paper. You only have to read one paper that Liz has been discussing in class, the Rodnina paper. The paper was published in 1999, OK? And it's still, I would say, a seminal paper. And what they propose or what you read about their model is still the working hypothesis in the field. But if you go and Google the ribosome in elongation, you will find out that in the last 10 years there are hundreds of papers now taking pot shots at this model using modern technological mechanisms, like single-molecule spectroscopy, Cryo-EM.

So they're flushing things out, but so still the basic model holds. So we continue to go through this because, in my opinion, all the machines that you're going to be talking about-- and this is part of the course-- have complex behavior like this with numerous substrates and many, many steps. And so hopefully one thing you got out of this paper is that kinetics are important.

OK, so today what I want to do, I'm going to ask you questions. I'm going to put some things on the board. We get you at talking points. I'm going to ask you some questions. And then the discussion will continue into the next recitation on the same exact same topic. But kinetics are important. But to do kinetics, what do you have to have? What's required to do kinetics if you look at this model?

So this is the model out of your paper. If you want to do kinetics, what do you need? Not only that, you have to speak loud because I'm deaf anyhow. What do you need?

**AUDIENCE:** To do kinetic studies?

**JOANNE STUBBE:** Yeah, to do kinetic studies.

**AUDIENCE:** [INAUDIBLE] a detection method in very controlled conditions.

**JOANNE STUBBE:** Yeah, so you have to have an assay, OK? And you'll see that everything you're doing over the course of the semester requires development of an assay. And I would say the more complex you get the more complex these machines. And that's what people are studying now as

opposed to if you looked at it Liz's lecture on tRNA synthetases, you saw a simple reaction, OK? That assay was developed decades ago.

But when you get into these more complicated machines, you have to be really pretty creative to develop an assay. And you need to have substrates. You need to get them from somewhere. And then you need to do kinetics. And so today, what I want to do is go through the kinetics part of this, asking you questions as we go along. And I'm going to start. So kinetics, in my opinion, is a key tool.

So we're using kinetics as a tool to study machines. And the machine we're studying is-- and have been studying is, is the ribosome. OK, so how many of you have had an introductory last lab course where you did kinetics? Only one? Two? OK, because steady state kinetics is where you start for everything, OK? And I find when I-- I've been teaching for many years-- that there are certain things about steady state kinetics that people don't seem to get.

And furthermore, were steady state kinetics important in this paper you had to read? Can anybody tell me? Did you get anything about steady state kinetics? Did you think about it? This will tell me how closely you read the paper. No? No one?

OK, so this paper is hard and this is a paper that, even though I read it probably 20 times, I still learn stuff every time I read it. So you can't read a paper once. There's huge amounts of information in this paper. And if you go back and look at it three weeks from now, you'll probably get a lot more because we're continually filling in pieces of information from you in this complex system. Yeah?

**AUDIENCE:** The part, I think, related to the steady state kinetics they measure Kcat, and Km, and their ratio.

**JOANNE STUBBE:** Right, so that that's where the steady state kinetics is. And so if it goes to the question of what can you learn from steady state kinetics, OK? So let me just put down a simple system, which you've all seen if you take an introductory biochemistry course. People use this system because you don't have very many rate constants. So when I write down rate constants, I don't put K's. I just put 1, 2, 3 because it becomes hard to read anything, OK?

So this is a simple system for any catalyst, OK, where some substrate could be EFTU, and tRNA, and GTP binding to the ribosome, OK? You do some chemistry to form some product. OK, and then the product dissociates. So if you look at the rate of the reaction-- so this

involves the assay. You have to develop an assay where you can monitor something as easily as possible. That's the key thing. So I think here is where your chemistry background plays an incredibly important role because you can be creative about your assays.

And so and you look at this as a function of the concentration of your substrate. What does the spectrum look like? What does the graph look like? How would you describe the graph? This is something you've seen in 507. We're just going back. What does it look like? Right, exactly-- rectangular hyperbole.

OK, and so I think what's important is that this kind of behavior has been observed over and over and over again since 1940s when this curve was first described by Michaelis and Menton with many variations on the theme. And so what you need to think about is you have two parts of the curve. What's happening up here? What is the dependence on the reaction on substrate?

So we have an enzyme that's a catalyst. It doesn't matter whether you're an organic chemist, an inorganic chemist, a biochemist. All of these things can be described by this simple, simple cartoon. So what's happening up here? What's happening in this part of your graph?

**AUDIENCE:** It's saturated.

**JOANNE STUBBE:** Yeah, see, you're saturated. So you're zero water and substrate, OK? And then what's happening over here? Your first order N substrate, OK? So from those observations, people derived equations, a general equation. So the rate of product formation, whatever you're assay is that you're using, is equal to Vmax times a concentration of substrate over Km plus the concentration of substrate. OK, so you've all seen this before.

And if you look at this one simple case, and you look at what is Vmax equal to-- can anybody tell me what are the rate constants within Vmax? So Kcat times the concentration of enzyme. OK, so Vmax, and what does that mean? Kcat we'll see in a minute, is the turnover number times the concentration of enzyme. That means all your catalysts have stuff on it. It can't go any faster. It doesn't matter if you add more, more, and more substrate. You have no catalysts. So that's what's limiting the reaction.

So if you derive this equation using steady state assumptions, what are the four sets of equations you need to be able to derive this expression? Can anybody tell me? What are the conditions you need to do? So what's the goal of deriving this equation, first of all? And then

what are the assumptions you make?

OK, so you want to be able to describe what you see experimentally, OK? So the first thing you have to do is be able to measure it experimentally, OK? So you have to have something in terms of an experimentally measurable parameter. And if you look at e, es, ep, which one of these are going to be measurable?

**AUDIENCE:** Going to be the substrate and the product.

**JOANNE STUBBE:** OK, so substrate and product. Yeah, you can measure substrate. You can measure product. But I'm talking about e. OK, so we have the e, we have an es, in this case we have an ep, most of the times you have 20 more e, equilibria. So which one can you measure experimentally?

**AUDIENCE:** [INAUDIBLE].

**JOANNE STUBBE:** Pardon me?

**AUDIENCE:** [INAUDIBLE] enzyme.

**JOANNE STUBBE:** Yeah, so you can measure the total enzyme. OK, so that's this the enzyme conservation equation. So you have-- I'm not going to draw this all out. This is a review that you've already seen, presumably. So that's the conservation equation. How do you measure the concentration of an enzyme?

**AUDIENCE:** UV vis.

**JOANNE STUBBE:** UV vis. What amino acids absorb in the visible?

**AUDIENCE:** Tryptophan.

**JOANNE STUBBE:** In the visible.

**AUDIENCE:** Oh, in the visible? None.

**JOANNE STUBBE:** None. So don't say UV vis. Say UV, OK? So this is key to being able to sort things out. So what are the amino acid side chains that absorb in the UV? This comes back to-- you need to--

**AUDIENCE:** Tryptophan, tyrosine.

**JOANNE STUBBE:** Right. So tryptophan and tyrocine are the major ones. Then phenylalanine is much smaller. So

you can measure this. But, in general, you can't measure all the other forms. OK, so you know this, and that's required to be able to get this expression that describes this rectangular hyperbole.

What about the substrate concentration? Under normal assays, if you've done an assay in the lab, how is the reaction set up? How much enzyme do you have in there? How much substrate do you have in there?

**AUDIENCE:** A lot of substrate.

**JOANNE STUBBE:** A lot of substrate. And what conditions are you under, perhaps, if you have a lot of substrate, over here in this graph?

**AUDIENCE:** You have to saturate your--

**JOANNE STUBBE:** Yeah, well, you don't have to, but you would be saturated if you had a lot of substrate. How much enzyme do you have in there? A lot or a little?

**AUDIENCE:** A little.

**JOANNE STUBBE:** A little, OK? So the enzyme, the concentration of the substrate is much, much greater than the concentration of the enzyme, OK? So that's a typical steady state assay when you go to determine the rate of your reaction. So because the concentration of the enzyme is much, much greater than the concentration-- the concentration of the substrate is much, much greater than the concentration of the enzyme, you don't have to worry about substrate being bound in these forms.

So the second equation you routinely use is called the substrate conservation equation, because it doesn't change, because the amount of this es on the enzyme, which is tiny, you don't need to measure it. So this is the second. So these are both conservation equations. OK, so we just said we were doing steady state kinetics, OK?

So now you need to be able to make the steady state assumption, which hopefully all of you know. So the rate of change of some intermediate with respect to time is equal to 0, that is we're under a set of conditions where the rate of formation is equal to the rate of disappearance of whatever this species is. And what is the fourth equation we need to be able to set up this? What is the fourth thing, which is probably the most straightforward? And, again, it needs to be in terms of experimentally measurable parameters. What are we

measuring in our reaction?

**AUDIENCE:** You said the position from vs to [INAUDIBLE] is irreversible.

**JOANNE STUBBE:** No, you I could have done this. And what would that have done to my equation? It just would have put in more rate constants. I'm going to show you what the rate constants are in a minute. There's nothing-- in fact, almost no enzyme reactions are irreversible. If you look, you can find reversibility in almost all reactions. This is-- so why do people write equations like this? They like irreversible reactions because it makes the kinetic derivation simpler. You don't have as many rate constants, OK?

So, but what do you need now? We're monitoring the reaction? What are you going to monitor? So we know how much enzyme we have. We can measure that. We know how much substrate we can have. We can measure that. We know what the steady state assumption is, and we have an equation. So we can describe that.

What's the other thing you need? It's the standard thing. How do you describe the rate of product formation? That's this guy over here. So what do you need? You need some kind of an equation that expresses just appearance of substrate, formation of products. So you need a way of measuring this. And you can do this many ways, even from a simple equation we've shown over here because a description of the rate of product formation is simply the net flux through any step in the pathway.

And so what you see people writing is they immediately go to an irreversible step because it makes the algebra simpler. So k3 times the concentration of ep would be the net flux through this step. But I could write the net flux through this step and I would get the same answer. So it's the net flux through any step in the pathway.

OK, so why am I going through all of this? OK, and the reason I'm going through this is because of this Kcat over km, which I just described to you. So one of the questions I asked you to think about when you're thinking about steady state kinetics is what are the two important parameters you get out of Michaelis Menton analysis?

And the reason I ask this is because, in my opinion, it's not correct in most textbooks. So what are the two important parameters you get that you learned about that you probably even evaluated if you did something in the lab?

**AUDIENCE:** Kcat.

**JOANNE STUBBE:** Kcat is one of them. OK, and what's the other one?

**AUDIENCE:** Km.

**JOANNE STUBBE:** OK, so this is what everybody says, is km. And that's not correct, OK? So let me put down what the-- what did I do with it-- the values for the kinetic constants are here. So in this particular simple equation, it's Kcat is 2 times 3 over 2 plus 3. OK, so this is Kcat. And Km out of this analysis is 3. The numbers really aren't important.

What I want you to see is that there are a huge number of first order rate constants in each of these parameters Km and Kcat, OK? Can you measure these? Can you measure these rate constants? That's what you want to know if you want to understand how this works, you would like to understand the reaction coordinate and what the rate constants are for every step in the pathway.

That's what the whole Rodnina paper is about with the long range goal of understanding fidelity. Can we come up with a model for fidelity in the translational process that's contributed by EFTU? So can we measure these guys from an assay the concentration of the enzyme, The concentration of the substrate, the steady state assumption? What do you think? Don't be afraid. This is a discussion. What do you think? Can we measure?

**AUDIENCE:** Does it depend how fast it is?

**JOANNE STUBBE:** No.

**AUDIENCE:** No?

**JOANNE STUBBE:** No. It is dependent on how fast it is, but it doesn't matter how fast it is to answer this question, OK? Anybody else got another guess? What? Your name?

**AUDIENCE:** Rebecca.

**JOANNE STUBBE:** Rebecca. What's your name?

**AUDIENCE:** Nicole.

**JOANNE STUBBE:** Nicole. OK, yeah?

**AUDIENCE:** Yeah. [INAUDIBLE] measure them [INAUDIBLE] we measure the initial rate, [INAUDIBLE] take that [INAUDIBLE].

**JOANNE STUBBE:** So you get Kcat and you get Km. That's not the question I asked. I asked, can you measure all the first order rate constants that make up Kcat and Km? No. So the problem with steady state kinetics is you can't really learn very much, OK? So what can you learn from steady state kinetics, and why do we keep looking at it?

OK, why is it the first thing you've seen this with the tRNA synthetases? You saw Kcat over Km values charging with valine or isoleucine, right? In this paper, if you go back and look carefully at the discussion at the end of the paper-- so hopefully after this class you'll go back and you'll read that-- a lot of the discussion is about mechanisms of fidelity where they are thinking about these initial steps.

And so these initial steps are really the selection steps of these things binding, OK? And if you go back and you look at the equation that they derive, it's amazingly complicated. Why? Because we have many more equilibria in our equation, but what you can get out of all this is Kcat and Kcat over Km.

So Km really is not very informative at all because it's composed of a whole bunch of first order rate constants. It's always never equal to the dissociation constant, OK? So you can't-- so what it is mathematically, it's the concentration required to reach half maximum of velocity. So it doesn't really tell you anything. It's just half maximum of velocity.

OK, so the two parameters that you need to think about-- and this goes back to the way you do experiments in the steady state versus the pre-steady state, which is what we're focusing on in this paper, is that you have two extremes when you do kinetics. And kinetics is something-- how do you learn how to do kinetics? You do them yourself. And you think about-- you think about what you think is going on.

And then you make guesses about what's going on. And these are one of the types of experiments, when you're doing them you change your experimental design in the middle of your experiment. So it takes a lot of practice to get good at kinetics. But what you do with all kinetics, look at the extremes, the limits.

So one extreme is the concentration of s goes to infinity. OK, so if you look at that extreme, what do you have? If s goes to infinity, what happens to b? What happens to this equation?

**AUDIENCE:** [INAUDIBLE].

**JOANNE STUBBE:** Yeah, so it goes to Vmax, or Kcat times the concentration of e. So you're up here, OK? And so what is Kcat? So you can get out of this Kcat. Why is Kcat an important parameter? Why do people care about Kcat? Hey, what's your name?

**AUDIENCE:** Alex.

**JOANNE STUBBE:** Alex. My nephew's name is Alex. I'll remember that, OK? You're stuck. What's Kcat?

**AUDIENCE:** It's like how quickly the enzyme turns over--

**JOANNE STUBBE:** Per active site. So it's called the turnover number. OK, so what does it tell you. It tells you how good your catalyst is, OK? So that's pretty important. So this is the turnover number. And I would also say it's pretty-- in the age of recombinant production or proteins, where we never isolate proteins from the normal source-- we isolate them all from bacteria or from yeast-- Kcat becomes really important to know, OK? So how do you know what the real Kcat should be if you isolate your enzyme from a protein that's expressed in E. coli? Do you think you get the real Kcat?

Have you ever thought about that? Most people haven't, OK? So you're not alone. What could happen if you expressed your protein in a bacteria, or in another model organism like yeast?

**AUDIENCE:** [INAUDIBLE].

**JOANNE STUBBE:** Yeah, it might not have the appropriate-- it's probably not-- it could be post-translational modification. It could be co-factors. So there are examples in the literature of very, very smart scientists who have spent 25 years of their life studying an enzyme that was only 1% active. So this is, in this course-- and I think in general in biochemistry-- you've got to go back and forth between the cell and what you see in the test tube.

So this Kcat, if the number is 0.00001 per second, you have to have some intuition that tells you, oh, my god. That's so slow. Something-- something is wrong. So this number of turnover is incredibly important. It gives you a feeling for how good your catalyst is. But the number we're really after is the second example and the other limit. And what happens is s goes to 0, what happens to this equation. So those are the two extremes, OK? So as s goes to 0,

OK, that's the other part of this equation. What happens to the equation? The rate of product

formation is equal to-- and I'll write Vmax as KT times the concentration of total enzyme, OK? I didn't write it down. Hopefully you all know that. So what you now get is Kcat over Km times the concentration of e times the concentration of s. So what is this guy, if you look at this equation? What's Kcat over Km? What are the units?

Kinetics isn't that hard. These are pretty-- if you think this is hard, wait till you start getting-- we're not going to go into derivation of steady state, pre-steady state analysis. But this is pretty simple compared to pre-steady state analysis. So what's Kcat over Km? What are the units?

**AUDIENCE:** [INAUDIBLE].

**JOANNE STUBBE:** Yeah. Yeah. So it's second order rate constant. So that's the key thing. So what are you looking at? You can look at that by this equation. What you're looking at is the enzyme combining with the substrate, OK? And that's what we care about. That's the specificity, specificity, or efficiency of your reaction. So if you have a tRNA loosing and an tRNA phenylalanine, they're both competing for binding to the substrate.

So the important parameter to think about that selection-- and that's why that's important at the end of this paper-- relates to Kcat over Km. It's the specificity or efficiency number. And if any of you ever work in a pharmaceutical industry, you'll find out that, of course, you never-- and you're looking for inhibitors, you never look at Kcat. Why don't you look at Kcat? You always look at Kcat over Km.

Why is that true? Can anybody tell me? If you were looking for a drug, if you were looking for an antibiotic, fusidic acid that we talked about today that inhibits the EFG that Liz talked about, how would you set up the experiment to look for inhibition? What would you do with your concentration of substrate? Do you want it high or do you want it low?

**AUDIENCE:** You want it low.

**JOANNE STUBBE:** You want it low. Why do you want it low?

**AUDIENCE:** Because [INAUDIBLE].

**JOANNE STUBBE:** Yeah, so if you're inhibitor is binding to the same site, and you have a huge amount of this, no matter what you do, even if this was a great inhibitor, if you had 10,000 times the amount of this, you're never going to see any inhibition. So understanding these simple principles-- which I can tell you there are people that don't get this in drug companies-- are pretty important, OK?

So Kcat and Kcat over Km, boring. But it's not really so boring. It's sort of central to everything that you'll be thinking about over the course of the semester and almost all the modules in some form, although we won't highlight it like we're highlighting it here.

OK, so, again, the reason we care about Kcat over Km is this question of selectivity. And I urge you to go back and look in the methods section of your paper. Now, this paper is packed full of stuff, OK? so as I said, I read it 20 times. Every time I read it, I find out something new. And furthermore, I think the paper-- how many of you found it a tough slog to go through this paper?

This is probably the hardest paper you're going to look at in my opinion? Did you think it was well written? Did you get the ideas? OK, Did you all get the ideas or not, or where you completely confused, or you didn't spend enough time on it? How much time did you have to spend on it?

**AUDIENCE:** Probably about an hour.

**JOANNE STUBBE:** OK, an hour. OK, so I would say-- I read the paper 45 times, and it takes me two hours to read a paper like this. OK, so again, it's a question of what level you want to look at things. And I think part of what this course is about is looking at experimental details. You're want to see that.

And the problems set, you're going to see that in lecture. You're going to see that probably next time when we continue to look at the primary data that they collected, how important it is to look at the axes, and not just looking at it rapidly. You really have to think about what the data is telling you. So this paper is complicated from my point of view because it's based on-- it's based on 15 other papers.

OK, so for you to really believe what they say, which is what you need to do as a scientist, how to critically evaluate somebody else's data, you need to really go back-- and we didn't ask you to do that-- and really critically evaluate the earlier experiments they've done, because some of the conclusions they've done, when we look at the primary data, I could have drawn-- without knowing all that primary data, I could have drawn a conclusion completely different.

So you see something and you've got to explain it, OK? And so when you start out, you have no idea. You have a very simple model. And in general, the model's almost always get more and more complex. That's what you're going to see over and over again. You start out as

simple as possible, and then things get more complex.

OK, so what we want to do now is ask the question. And I've just told you, you can't evaluate these individual rate constants. We just don't have enough variables, OK? We don't have enough that we can measure, that we can change the substrate concentration we can change, which changes the rate of product formation. So those are the two variables.

But we have many more unknowns. We have k1, k2, k minus 2, k2, et cetera. So we can't evaluate these things. So the question is, is there any way you can start getting the primary rate constant, the numbers to the primary rate constants, OK? And so one way that people do this nowadays-- and when this paper was done, this was not an easy task.

OK, now because of molecular biology where you can get large amounts of protein, it has become much more of an easy task-- you can get a large amount of protein-- you want to turn to the pre-steady state. So what I want to do very briefly is discuss the pre-steady state. I asked you to think about-- I asked you draw this out. This is one of the talking points in the questions I handed out.

But in the steady state, we're over here. And the pre-steady state is before we get to the steady state. And does anybody have any idea what timescale you are on in that region of the curve? Is it hours?

**AUDIENCE:** Milliseconds?

**JOANNE STUBBE:** Yes, so it's milliseconds. So, fortunately, this didn't necessarily have to be true-- most enzymatic reactions occur. the catalysis occurs in that time regime, or maybe 0.1 milliseconds to milliseconds, allowing you to be able to use this method in an effort to try to understand what these-- evaluate what the rate constants are.

And when you look at the table in the Rodnina paper, we're going to talk about where all those three constants came from, OK? Are they good or are they not good? But that's what you'd like to know for every system to really understand the question of fidelity, whether it's translation fidelity, DNA fidelity in replication, transcriptional fidelity.

And you'll even see in Liz's section on polyketide syntases, which make natural products, you also have fidelity issues almost everywhere. So you'd like to be able to evaluate these things. And you can get a handle on this if you're a chemist and really care about the molecular details using potentially kinetics. So this is why kinetics is one of the first places that you

actually start to think about what's going on in any reaction.

OK, so let's say a few things about pre-steady state. I'm going to ask you a few questions, if I can remember what I'm going to ask you. OK, so OK, so let's suppose in this simple case, which I just covered up, this step is rate limiting, OK? So what is that step? Do you think it's common that a step like this-- so we have e plus s. And eventually, the enzyme gets recycled.

I'm saying this is the rate-limiting step. Where is the chemical steps? Where are the chemical steps in this reaction?

**AUDIENCE:** 2, 2.

**JOANNE STUBBE:** Yeah, so k2. What are these steps over here, k1, and k minus 1, and k3?

**AUDIENCE:** It's like association of the--

**JOANNE STUBBE:** Yeah, so the physical steps, OK? So as a chemist, and you're trying to understand what's going on, isn't it a problem if the rate limiting step is physical? It masks all the chemistry, OK? So what you see in this paper is they have to figure out clever ways to get around these kinds of issues.

And you see that over and over again when you're studying enzyme systems because enzymes have have had billions of years to evolve. They are evolved. Their catalytic transformations are amazing. They go 10 to the 15th per second, right? That's totally mind boggling. Chemists can't come close.

And so what happens then issued a limited by physical steps. So what we want to do is try and then look at the first part of this transformation. And basically, what we're then doing is using the enzyme sort of as a reagent. There are numbers of ways you can do this so that you can have a way of not looking at multiple turnovers because you can only look at one turnover if this is blocked in terms of product release.

OK, so I think this product release is quite often the rate-limiting step in biological transformations. And what have you seen from reading the Rodnina paper? Have you seen conformational changes in thinking about the kinetic model we had up there before, and Liz had on the slide? Have you seen conformational changes? Is that part of the mechanism? Are they fast or are they slow?

**AUDIENCE:** Wasn't that part of their reasoning that the difference between if you have a cognitive versus if you have a mismatched? That influences the rate of the reaction based on how it can affect the conformational change?

**JOANNE STUBBE:** Wait, so that's exactly what's going to happen. So there are multiple places. How are you going to discriminate between two amino acids? Cognate and near cognate, whatever they are, will get to the data. The question is, how do you do that? And one of the steps is-- we talked about today GTP hydrolysis. But GTP hydrolysis is limited by a conformational change.

And then once that go, the hydrolysis is very fast. And so it looks like the rate constant for GTP hydrolysis is the same as the rate constant for the conformational change. Where else have we seen a confirmation change the accommodation?

**AUDIENCE:** Peptide.

**JOANNE STUBBE:** Yeah, so peptide confirmation. This is shown here is this little cartoon where this red ball is the amino acid. It needs to reorient itself so it can form a peptide bond. So confirmation changes are all over the place in entomology. And if you look at the ribosome, do you think it's easy to tell with those conformational changes are from a molecular point of view? What do you think? Do you think it's easy or hard?

**AUDIENCE:** Hard.

**JOANNE STUBBE:** Very hard. OK, so here-- one of the most amazing things about the ribosome-- you've got to think this is amazing. You have this called the anti-codon loop way down here on the [INAUDIBLE]. And the GTPase is 80 angstroms away. And somehow, twiddling-- you saw this in class today-- these guys to form the right confirmation is transferred 80 Angstroms.

And that triggers the reaction, rapid and irreversible. And the reaction goes to the right. You see this over and over and over again in these machines. OK, so this is a really important observation. How does that happen? Well, I think what's mindboggling about the ribosome-- again if you Google ribosome and elongation, you'll see we have another 150 papers published where people are trying to sort out-- because we have cryoem structures, we have stagnant crystallographic structures, we have single molecule stuff now.

On top of all this model we have from Rodnina, people are trying to sort all those out because they care about how it works in some detail. So who ever would have thought we could get to the stage where we-- you've seen the pictures you saw in class today. Those pictures-- when I

was your age, do you know how many structures there were? Maybe three.

OK, we had hemoglobin. We had chymotrypsin. There were no structures. And why was that true? Because we had no molecular biology. So I used to spend three-- I'm digressing. This happens to me all the time. You're going to hate me for this. I'm going to get hammered for this. But I used to spend three months in the cold room isolating a microgram or protein, OK?

And then molecular biology came in. And it's still not easy. And Liz will tell you what the issues are with purification of protein. But you can get grams of protein now in a day, OK? So there's been a revolution. And that allowed these crystallographic-- a pure material that crystallized more readily. And then the technology on top of that has really revolutionized what you can do.

I think it's a very exciting time. And I think any of you who want to be biochemists, or are thinking about drug design, you really need to learn how to look at structures. So that was the first module. It takes a lot of practice. You need to figure that all out.

OK, so pre-steady state-- so we're going to look at pre-steady state. And the goal is to evaluate the individual rate constants. OK, so that's the goal. And you may or may not be able to achieve this goal. But this happens, we just said, on the millisecond timescale. And so one of the questions-- and we're doing this under single turnover.

OK, so let's look at a simple-- and we've just talked about it in the steady state. The concentration of the substrate has to be much, much greater than the concentration of the enzyme. And the enzyme concentrations are really low. So let's say we have an enzyme concentration of 0.01 micromolar, OK? So that's our enzyme concentration. And that would be typical if you would be using in a steady state assay if you have done those.

And let's say that we're going to monitor this reaction by some kind of absorption change, a unique absorption change. So we're looking at absorption at some wavelength, OK? And let's say the extinction coefficient for this is 10 to the 4 per molar per centimeter. It would be ATP or coA.

Then you can ask yourself the question, under these conditions, the change in absorption at whatever this wavelength is, is equal to the path length of light in centimeters times 10 to the minus 8th molar times 10 to the fourth molar per centimeter. OK, so what would your change in absorption be if you were measuring this in a single turnover? It would be really, really small, 0.0001. Can you measure that?

Maybe you could measure this if you took hundreds of samples and you did a statistical analysis on it. But it's really low. So what do you want to do then to do pretty steady state? What's the thing to change so that you will be able to see something?

**AUDIENCE:** [INAUDIBLE].

**JOANNE STUBBE:** Yeah, so you increase. So when you have this, and you can't see something-- and, obviously, it depends on what this extinction coefficient is-- but this is a pretty high extinction coefficient. So what you do is you increase the concentration of enzyme. And if we increase it, say, 1,000 fold, then then so we're now at 10 to the minus 5. Then now what is the change in absorption? The change in absorption is 0.1, which you can measure fairly easily in any kind of current instrumentation.

So the thing is you have to be able to see. So the key thing with pre-steady state, and the reason you need to have large amounts of enzyme, is you need to be able to see what you're monitoring. So it's all about sensitivity. You need to see. And this usually implies increasing the concentration of the enzyme.

OK, so what's the problem if you increase the concentration of the enzyme? Say we normally are at 0.01 micromolar steady state. We now are at 1,000 times higher. What's going to happen that makes the analysis complicated? If you increase the concentration of the enzyme, what does that do?

**AUDIENCE:** You're going to burn through--

[INTERPOSING VOICES]

**JOANNE STUBBE:** You're going to-- yeah, it increases the rate of the reaction because the rate of the reaction is proportional to the concentration of your catalyst. If you don't remember anything else out of this course, or anything in chemistry, that's pretty important no matter what area of chemistry you're in. So now what happens is the reaction is going like a bat out of hell instead of pipetting by hand.

By the time you pipetted and put this into however you're observing it in the spectrophotometer, reaction's over, OK? So that's what the issue is, OK? So the sensitivity is key. And then the second thing you need to think about-- so sensitivity is one thing. And the other thing you need to think about is, what are the limitations of this method? How fast can

the rate come-- on the millisecond timescale, what are the limitations in terms of the rate constants you can actually measure?

So when you're looking at these reactions, you're looking at, in general, first order reactions. So all of these take place on the enzyme. So everything is stuck on the enzyme. So it's all first order. So the half life of the reaction is, if you go back and you look at your introductory kinetics, is 0.693 divided by k observed.

And so if you had, say, a rate constant of 500 per second, then that gives you a half life of 1.5 milliseconds, OK? So that means you have to be able to make your measurement faster than that, OK? So the instrumentation we're going to be using can't do that. So the instrumentation we're doing-- so this would give you a half life if you calculate this. I don't even remember what the number is.

But the dead time of the instruments that you would be using to make pre-steady state kinetics is approximately 2 milliseconds. So by the time you could stop looking at the reaction in some form, you know more than 50% of the reaction is gone, OK? So the rate constant then limits also what you can measure.

So we asked this question before-- how could you modify this rate constant? What could you actually do? How could you make it so you might be able to say your rate consent was 500 per second-- you missed more than half your reaction. What could you potentially do so that you could monitor more of the reaction? What's the parameter that you would change? Kinetics. Think about kinetics. What do you always control in kinetics?

**AUDIENCE:** Substrate concentration.

**JOANNE STUBBE:** OK, you can control substrate concentration, but that's not the one I'm looking for.

**AUDIENCE:** Temperature?

**JOANNE STUBBE:** Temperature. Yeah. So in our body, we're at 37 degrees. That really is where you want to be making all of your measurements. In reality, many of the measurements are right on the edge. And so if you read the papers carefully, you'll see that people do lower the temperature, and that the rate of the reaction is related to the temperature.

What's the problem with lowering the temperature? These are all things just you got to think about in the back of your mind. They're all playoffs in terms of how bad you want your

experimental data and what the issues are with interpretation of data. Yeah? Rebecca.

**AUDIENCE:** It makes it difficult to compare the different values. And you might not know exactly what the relationship between temperature and rate is, like if scales linearly.

**JOANNE STUBBE:** OK, so that's true. You could have a huge conformational change that doesn't have erroneous behavior. I think quite frequently, most enzymes, they're designed to work at 37 degrees. And when you start cooling them down, they do weird things. So you could make the measurements, but then you have this issue-- I think, which is what you were saying, of how do you extrapolate that? So a lot of times you will change the temperature because that's the only way you could make the measurement. But the caveat is, like with everything, that you need to think about what the consequences of that actually are.

OK, so the methods that we're going to be using Liz already introduced you to in class, not today, but the previous time. And so what you want to do, since you can't pipette on the millisecond time scale by hand, you want to have an instrument that allows you to control the rate of reaction by-- you put two different things in your syringes.

And then you have an instrument-- push the two syringes into a chamber where they're mixed. Do you know what the rate-limiting step in this process is? It's the mixing process, OK? So the mixing processes is 2-millisecond dead time. I don't know if any of you have ever mixed something viscus with something not so viscus? What do you see?

Have you ever made up a solution of glycerol? No? They probably give you all this in a kit nowadays. You don't have to make up your solutions of glycerol aluminum anymore. So this goes back to experimental design. And I'm not here to teach you how to do experimental design.

But if you had very high concentration of the enzyme-- because we need a lot to be able to see something-- and you're mixing it against substrate-- you have something very viscous and something not viscous-- and when you mix them it takes a lot longer to remove all the lines from the mixing process. So experimental design, you really need to do some thinking about that. Once it gets into the mixer, the liquid in the mixer pushes a third syringe back. It fills the syringe up with liquid. It hits some kind of a stop position, which then triggers detection, OK? So that's what you're looking at.

And the beauty of this method is it's continuous. And so what do you have to do to be able to

look at this? What did they do in the case of the Rodnina paper? What kind of method did they use? Did you think about that? They described it, but you might not have thought about it in terms of experimental design. How did they monitor their reaction, one of their reactions?

**AUDIENCE:** [INAUDIBLE].

**JOANNE STUBBE:** Yeah, so they are going to be able to somehow tag-- and this is a key thing, is how do you tag something in the right place so you can see a unique fluorescent change? That's not so easy, OK? So you mix this. You can monitor this continuously by change in fluorescence. If you had something that has a visible absorption, could you use that? What would limit that?

Say, if you looked at tRNA synthetases that you talked about in class two times ago, where you were looking at ATP that isolates the amino acid to form the adenylate, which then reacts with the tRNA, could you use-- ATP as an absorption at 260. Could you use that absorption change? Do you remember what that equation is?

Do you remember installation of amino acid? You're going to see this again in polyketide syntases. It's used quite frequently in biology. Nobody has any idea? Nebraska, how about you?

OK, so here we have amino acid plus ATP. I'm digressing. But if you learn this part, you've learned something out of all of this, that forms the acyl adenylate. OK, how did they monitor this reaction? You discussed this in class. How do they monitor this reaction?

**AUDIENCE:** [INAUDIBLE].

**JOANNE STUBBE:** You need to talk louder. You need to be assertive, OK?

**AUDIENCE:** [INAUDIBLE].

**JOANNE STUBBE:** Yeah. So we're going to talk about radioactivity next time. This will be one of the methods we're going to be introduced to. Why couldn't they use ATP?

**AUDIENCE:** The absorbent's different between [INAUDIBLE].

**JOANNE STUBBE:** Yeah, they're the same. Yeah. So you have to have a difference in absorption to be able to measure the visible. So the total absorption is due to the adenosine moiety which is the same in both molecules. OK, so you can't do that, OK?

So that's one. And then let me just do one more thing, and then we'll quit. I still have another minute according to my watch. OK, so the second method, which they also used in the Rodnina paper is rapid chemical quench, OK? So, again, you have two things. You mix them. There's some plunger that allows the mixing.

And then this is a discontinuous method. So what happens is you mix. And then you have to stop the reaction, OK? So you have a third syringe where you mix in something to stop the reaction. And then you have to analyze what comes out the other side. And this is where they're going to use radioactivity. And so this is rapid chemical quench.

And how can you monitor a rapid chemical quench experiment? What's the best way to stop the reaction? What did they do in this paper? How else could you stop the reaction? Anybody got any ideas?

So what what's the first criteria if you're going to stop the reaction? What does it have to be able to do? You just can't throw in anything, right? What is the key criteria for successful stopping?

**AUDIENCE:** Something that will turn off the catalytic activity?

**JOANNE STUBBE:** Yeah. And it has to be able to do it on a millisecond timescale. So you need millisecond stopping. OK, how could you millisec? How could you stop something on the millisecond? What would you use? Anybody got any ideas? So this is not trivial, actually.

**AUDIENCE:** You could change the pH?

**JOANNE STUBBE:** Yeah, so changing the pH. But so you could go acid or base. Acid works. In general, base doesn't work. So if you read the handout that I've given you, it does work. But it's much, much, much slower. And every base is different. Acid works all the time.

There's another thing that you can use that is quite frequently used, especially with polymerases that work on nucleic acids. And that's EDTA. So this is a chelator and EDTA chelates the metal, which is essential for viability. So that also-- the chelation can occur in the millisecond timescale. So what we're going to do next time, I've sort of introduced you to the pre-steady state.

The next time we'll come in. And we're going to look at the actual experiments. We'll look at fluorescence. We'll look at radioactivity and how you measure radioactivity. We're going to look

at antibiotics, like you talked about today. We're going to look at non-hydrolyzable GTP analogs.

If you look carefully at this paper, it's amazing how many methods they used to come up with this model. And that's one of the take home messages that you have to use many, many methods. And then you never get an exact solution to your equations. It's numerical integration of all the data that leads you to the model that they've actually used, OK?