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Speaker: Clifford Unkefer

Note: Transcript has been edited for clarity, but has not yet been reviewed by the author. Accordingly, inaccuracies may be present. The author-edited version of the transcript will be posted at a later date.

Clifford Unkefer: I'd also like to thank Claude for inviting me. We're tied together, actually, by our funding agency. So we both have research resources from the NIH, and funded by now the National Institute for Biomedical Imaging, and Bioengineering, or NIBIB. And (that's) a strong statement to me, actually, because our program manager sort of moved us two together. So I'm here to actually participate in this, and hopefully develop new users. And when I'm talking about stable isotopes, it's a limited range of these light isotopes of (deuterium), carbon 13, nitrogen 15, O17 and 18, (and) the ones that you use to study biological systems.

By way of introduction, I want to say one other thing. I'm one of those color blind guys too. And you can tell after awhile because you'll notice that the people who have it really bad only have white, black, and gray clothes. That's what I have. Anyway, so I think there won't be any issues with color in my presentation. Okay.

So what I really wanted to do is tell you a little bit about our synthetic capabilities. The properties of these isotopes, and how they've been used to study metabolism by other methods, so we can learn those lessons, and not have to relearn them, as happens often when people start detecting from when they went from radioactive detection to (NMR) detection (Slide #2). We've learned a lot of lessons, and I think we should try to – when we start detecting with MIMS, try to learn from that, and not repeat those mistakes. And this is the idea that I want to introduce that Claude was talking about is sort of correlated labeling, which will let you get at more deep metabolic questions. And also I'm going to talk about synthesis, a little bit more synthesis of the compounds we're trying to get at. Unfortunately, we haven't – it's my fault we haven't finished all of our chemistry, so we don't really have any MIMS images to show you yet from my label compounds.

One of the first lessons is – that in stereo chemistry and biology is very important (Slide #3). For example, amino acids like Claude's used N15 amino acids, it's important that you have only the isomer. And this dates many radioactive and stable isotope labeling experiments where people put in DL mixtures because they're easy to synthesize. They'd have a single label. But the label doesn't trace the stereo chemistry. And what happens when you do this, the natural isomer's actually diluted in the biological system by the compounds that are being made.

So effectively what you've done is created the one you want to trace the metabolism of there in sort of a low specific activity, or a low labeling; and a very high specific activity label in the form that you don't want to look at. And people have made mistakes because they end up tracing the metabolism of this one because this one's diluted by the natural compound. So we go to great efforts to do stereo chemistry.

This is just another example to show you one of the classic ones is people made C14L (carantine) (Slide #4). Mixed it – or they made DL carantine that was C14 labeled. Mixed it with L carantine, and fed it to cells. And they wound up tracing the radio labels from the D carantine, and never really seeing what was going on with the natural metabolism. The thing they were trying to trace.

So we go to great lengths to do stereo chemical pure synthesis. And I just want to give one example where we've spent a lot of time focusing on amino acids actually for structural

NMR studies. So some of this (will be) presented in those terms. And one compound that we spent a lot of time labeling is (valine) and (lucine) who have these (diastarytopic methyl) groups (Slide #5). And what want to do is actually differentiate them by isotope. So not only did we have the natural stereo chemical center to take care of it too, we created a new stereo chemical center by introducing labels. So you had to take care of it there too.

And when we were trying to label valine for NMR – it doesn't show up very well, but you can see that the methyl groups, there's not much dispersion in the chemical shifts of the protons, but there's a great chemical shift dispersion in the carbon, or a much bigger one (Slide #5). So we wanted to have C13 labels in both of these positions, but then differentiate these methyl groups by the number of protons and deuterons that are there. I like telling the story, because (it lets) us learn a lot of new chemistry.

Anyway, this was the target molecule. You wanted to have, again, a C13 label in blue here. And then differentiate these methyl groups by either having two deuterons, or one deuteron. And again, maintain a stereo chemistry at this point.

Hojatollah Vali: How do you get one – the left, and not the right?

CU: I'm going to go into that. So. We do it by (kyralaxilaries), or else we use enzymes in the synthesis. Those kind of approaches. Organic chemists are now, they're getting in the realm of biology to greater than 99 percent one isomer or the other, if they do things the right way. So we'll go into that a little bit.

So the first thing we had to do is actually develop methyl groups that had one deuterium, and two protons, or the other way around. And there was this one paper that came out, actually, from another research resource, the one on (tritium) labeling, where they generated methyl (iodides) that we were ultimately after with a single tritium on it by reducing off this chlorine. This gave us an idea about how to do this, and we developed this method where, again, we can go into this more, but everything we make starts from carbon monoxide. It makes the chemistry hard. And we have to build all of our carbon skeletons one carbon at a time. So we make methanol, and we can make deuterated methanol. We generate methyl iodide in situ, and then make this (zioanosol). So you've actually sort of trapped this methyl group in this high boiling molecule on which you can do some chemistry. And what we found is that if you can treat this as a very strong base, you get to extract one proton. If you do it very carefully, you extract only one proton, and then we can add D2O and (deuterate) that position. It doesn't show up very well, but this is a C13 spectrum of just the methyl group (Slide #8). Now it's coupled to the (spin one). We decouple protons, and it's coupled to the spin one deuterium, so you get three lines for each one.

You start with deuterated methanol you get methyl groups that have a single proton by extracting one deuteron, and then adding a proton back with water. Now you get – you couple the two spin one nuclei, and you get this five line pattern (Slide #8). Didn't show up very well. But anyway, these are pure molecules now. One proton, two deuterons, or two protons, one deuteron.

And the next step is how you take care of the stereo chemistry. This is a (kyralaxilary). This was developed by a Swiss chemist named (Upholser). So this is often referred to as Upholser chemistry. And what you can do is add a molecule to this kyralaxilary that's based on the natural camphor molecule. You can obtain that in (high) stereo chemical purity. Then you can extract one proton with a strong base like ?? (lithium), and then alkalate the (enalate) that you generate there. And that generates a stereo chemically pure molecule. And since you have (diastaryomers) now, the chemical shifts of these two methyl groups are different. That's because of the stereo chemistry here.

So we can analyze this while it's still on this molecule very carefully by NMR. Very easily by NMR. And we see C13 in one position, versus the other. This is a methyl group region of that carbon spectrum. You see actually four methyl groups for these two, and then these two on the kyralaxilary (Slide #9). If we add C13 label, we see only one of these (peaks) getting labeled. You see unlabeled small bumps left for the other three methyl groups. These two from the kyralaxilary, and this one, and this one are the two methyl groups here. So the labels only go into one position.

So this just shows our whole strategy for then making the carbon skeleton for valine (Slide #10). We don't want a deuterium in this position, so we had to start with deuterated acetate. We alkalate it once. We generate (enalate), alkalate it once with one of our IS2 methyl groups. And then we alkalate it again, generate the stereo chemical center with IS1 methyl 1 with a single proton on it.

What we have to do to make valine is to go on and add one more carbon. So it turns out we have to knock it off this kyralaxilary, add another carbon, and then stick it back on kyralaxilary. So that last part is shown here (Slide #11). We add a single carbon. Now we have the backbone skeleton we need for valine. We stick it back on the kyralaxilary. And then there's an (electrophylic amanating) reagent that Upholser developed, and that we've (made labeled) now so that we can add this nitrogen from the bottom face also to generate an amino acid that's pure in its stereo chemistry at both of these centers.

And just to show you that this works as a 2D proton, spectrum proton, carbon-correlated spectrum of the two methyl groups (Slide #12). This is without deuterium decoupling. And we see again the five line pattern for one of the methyl groups, and three line pattern for the other. Now you can decouple the deuterons, and then you can see both of these as single peaks. The reason we made it was to label proteins so you could do NMR, and proteins that have high molecular weight. This is 35 (kiladalton, halall). (Can be halaganase). It's 310 amino acids, has 14 valines in it (Slide #13). So we labeled it using an (oxotropha V coli) that required valine. So we only needed 20 milligrams per liter of labeled valine. And this is also on a (per-deuterated) background. So we grew completely on D2O, and with deuterated glyserol as a carbon source.

This is a 2D NMR spectrum of the valine methyl region (Slide #14). So the only thing with any C13 are methyl groups. So this is, again, protons correlated to carbons. You see the carbon chemical shifts spread this way, and the proton chemical shifts spread that way. These are the only carbons that are labeled, so the only things that we're seeing here. Now if you didn't do this kind of labeling, you would see, in this large of a protein, no resolution in any of this region; it would all be a blur of (resonance) that you could see. So this is much bigger than

proteins that have been reported. Interestingly, you can actually make the stereo chemical assignments – actually, if you count these all up, first of all, there's 28 peaks like you'd expect for 14 valines. You can make the stereo chemical assignments because it turns out the methyl group carrying a single proton is sharper in both the carbon and the proton dimensions. So you're getting these much sharper spots, compared to the bigger spots for those that carry two protons and one deuteron. And you can use some of the spin selection methods to actually turn off say the IS2, versus the IS1. So we selected for those that have an odd number of protons on them, and we see just the sharp peaks, the sharp 14 peaks for this methyl group. In the same sample we just turn this off by an NMR (trick). Sort of our target. So anyway, we do take care of stereo chemistry. Probably beaten to death here.

I wanted to talk a little about the properties of these isotopes (Slide #15). And there's the important ones for doing MIMS, and then there's the important ones for getting the experiment done. So deuterium is obviously (cheap). It actually has low abundance, but it has some other problems that we'll go into, in terms of metabolism. It's readily available. This is essentially water distillation made very inexpensive by the fact that it's done mostly by power companies now. C13 – Nitrogen 15, O17 and O18 are all separated by the same process, at least at Los Alamos, and also in the private industries in this country. And that's by distillation of either carbon monoxide to get C13, or nitrogen oxide, which gives you the nitrogen, and the oxygen isotopes.

So the CO distillation column at Los Alamos is 700 feet long. It goes down into the ground. It's a cryogenic system that's cooled to liquid nitrogen temperature. CO is a condensed liquid there. You distill it through that 700 foot long column, and what you get is mostly depleted C13 coming out of the top of the column. At the bottom of the column you've got a mixture of C13, O16, and C12, O18. And the enrichment that they obtained at the bottom of the first stage was 90 percent. So then what they have to do is bring it back from the bottom of the column up to the – this is all done in a large well hole in the ground. So they bring it back up to the surface, and they heat the CO to 1000 degrees to scramble the carbon 13 and the O18 label. So now statistically most of the O18 is (on a) C13. Then they redistill it, and that's how they get to 99 percent.

The NO process actually is a – there's an isotope effect. It forms (dymers) in the liquid phase, and there's a big isotope effect on that. So it turns out that in a single stage you separate mostly isotopomers that are N15, and O18, or N15, and O17. And it's a much shorter column; only 200 feet long. Because there's a big isotope effect on that. And the separation factor in the distillation is a factor of 4 or 5 greater. So they don't need as long a column. Okay. And out of this we get the high enrichment O18, and occasionally a fraction that has O17 also.

HV: ??.

CU: Yeah. Sulphur is (separated), potentially distillable, but we never got to that at Los Alamos. So these are separated, at least in the DOE complex, by the (calutrons). And it's very expensive. I didn't even look up the price, but it is available, and it may be possible to do sulfur isotope experiments also. So I want to go through each of these now again.

Deuterium's cheap (Slide #16). It's also available in the precursors that you need to do chemistry. Readily available from many commercial outfits as deuterium gas, deuterated water. And there's also a bunch of (equivalent) to deuterium gas chemically. A bunch of (hydrites) that you can do reductions with. But it has two disadvantages, and one is that the label can wash out during the metabolic transformation. So you might not predict. You have to be careful about it.

And the other, probably worse, effect are these large kinetic isotope effects that come with deuterium. So you perturb actually what you're trying to study if you use deuterium.

XX: ??.

CU: I'm going to show you right now. So – well, actually, this is the kinetic isotope effect (Slide #17). So we'll go on. Okay. So deuterium will equilibrate in some kind of chemical – some chemicals by equilibrating with water (in the cell) in 55 molar. So these reactions may be slow, but there's a big driving force, because the water's there in such high concentrations.

So, for example, Claude used very successfully and very elegantly in 15 labeled amino acids to watch proteins. He could have selected deuterated amino acids. That would have saved him a lot of money. But there's a problem because, say, for alanine, for example, it's (transamanated) to make this (alphaketo) acid (pyervate). And pyervate can undergo this (tautomeric) equilibrium. So you can write this (tautomer), and what happens is in the formation of this is hydrogen exchanges with water. And, of course, the back exchange causes you to lose this deuterium. If you go through three times you'll wash all of the deuterium out into the water in the cell, which gets pumped away. You have to be careful about whether there's exchange. And it may not be as obvious to you. Any time it's next to a ketone or a (carboneal) there's a potential for exchange.

And this is actually pretty fast. People have observed this doing NMR where they're actually just C13 labeling here, but they're growing in DTO, or they're growing – what happens is alanine may have a proteated methyl group to start with, but because it's being – the culture's grown in the presence of DTO you get exchange in the other direction. And (in) NMR they see all the isotopomers of this methyl group. So this is observed readily, and it's a relatively rapid process, compared to, say, growing cells over days or weeks.

So you have to be careful about deuterium, and just go back. These are the kinetic isotope effects (Slide #17), so any time there's an enzyme reaction that actually involves breaking the carbon/hydrogen bond, these isotope effects for deuterium are pretty big. They can be a factor of – they're commonly a factor of 4 or 8. They've been observed as high as 30, which wasn't predicted for any kinds of chemical reactions. So you may perturb, actually, the very metabolic regulation or the metabolism you're trying to observe.

And this is – I don't have a good example referenced here, but there's lots of examples in the literature where people use deuterated drugs, and they try to measure the metabolism of them by feeding a deuterated drug to a whole animal, and then analyzing for metabolites in the urine. And it turned out because the first step was, say, a mono-oxygenase that was going to insert oxygen in a particular bond that they deuterate with deuterium, they slowed that reaction (real

fast), by a factor of 4 or 8, and they sped up the hydroxylation, say, at another position. And it turned out the deuterated drug was metabolized by a whole different route than in the (proteo) drug. So you end up studying sort of the wrong metabolic transformation. So this is something you have to be real careful of (Slide #18).

Okay. So we see the deuterium chemistry is relatively easy. It's another reason these compounds are cheap. So (I) often take advantage of the same kind of reaction we were just talking about. You can exchange deuterium in, say, if you want to make deuterate glycerol. Start with the hydroxy acetone, and then the other kind of reaction are these reductions. So you introduce the second deuterium, and trap these into glycerol. So this kind of chemistry's fairly straightforward and easy to do, but unfortunately, deuterium's not the best.

So go back, I think, to nitrogen 15, again, it's relatively available (Slide #20). It's on the order of $1,000 to $2,000 per (mole). It's available in high enrichment forms; 99 percent. It's also – like I said, it's separated by cryogenic distillation of NO, but there's good chemistry involved to make it – because they cover the oxygen 18 out of the same molecule, they convert it to ammonia, and also to nitrite.

These are the two most important forms for incorporating into organic chemistry, because you can either do (nucleophylic) substitution, say, of (halites). You can use ammonia, or a derivative we've introduced, which is a (flexinomid), which lets you do these displacements, and know that there's only one (bromated) compound going on there, and you have to hydrolyze this. This is one route to amino acids that are readily available with N15. The other one is this electrophylic amanation (Slide #21). These two reagents are well known in the literature, and they come from nitrite in a single step. So they're available.

Again, as Claude has used, there's commercial N15 L labeled amino acids (Slide #22). We make those by this Upholser chemistry, again, where we can start with any (ACL) group here to make any of the 20 amino acids, and add this nitrogen 15 selectively, (face) selectively so you get just the right stereo chemistry here. And then actually we've done a lot of work on this transformation. And I can tell you that you cannot detect the (de-isomer) if you're making an L amino acid here by any method that we've been able to do.

You have a problem when you (saponify) off this (kyralaxilary). If you're not careful you'll actually introduce some of the – (racimize) some of the amino acids. So you have to be very careful about this last step of (deblocking). You can certainly generate this quite stereo chemically pure. Certain amino acids are more susceptible to racimization in this step. So some of them don't come out as pure (Slide #23).

Commercially there's a reason that most of these are available, and especially the lucine, because this was developed directly by this by (Ajami and Yan) actually in Cambridge. They start with an (aldahyde) that's one carbon short of an amino acid. They can either make one C13, or N15 amino acids by starting with CO, or (acidum), this (amid) of acetic acid, (acidumid). It's N15 labeled. The product then is an N15, (anaceteal) amino acid. And it's a racimate. It's DL because there's no stereo chemistry here. But then they go on a second step. They use an enzyme, (honkadeacelase), which will knock the aceteal group just selectively off just the L amino acid.

Then you create a mixture that's the D amino acid acetelated, and the L amino acid that's free. And that's why these are readily available, and relatively cheap molecules. So the ones where there's readily available aceal groups like lucine, and (fenelalanine) are commercially available at reasonable prices.

Okay. And I just wanted to show another one of our examples that we've developed routes to label purines (Slide #24). And we can actually put nitrogen 15 in any of these positions. And this is a common route which goes through this common (chloropurine) intermediate that will lead you both to G or to A. You develop the (perimidine) ring from urea, which you can label with N15 easily in both positions. That's N15 there. We react with – actually, we make this chloro-derivative, and then react with ammonium hydroxide, or ammonia. Put nitrogen 15 in that position. We can then add a nitrogen from one of these electrophylic amanating agents in that position. Close this ring to make the chloropurine common intermediate. And then we develop routes to get both to A or G. We can put all the labels here.

This first step actually tells you pretty much how we make perimidines also, so we've made those labeled, and recently supplied Claude with N15 labeled (thiamine) made from urea the same way. The nucleotides are available.

(To) go into this a little bit more. The elegant experiments that Claude did with N15 to look at protein turnover make some assumptions that as a labeling chemist I'd like to figure out a way to get around. And that's the fact that nitrogen 15 does get passed to different amino acids. So if you start by labeling with N15 in (fenelalanine), then you'll transamanate with other (keto) acids, for example, (hyrovate). You'll transfer the label from fenelalanine to alanine (Slide #25).

Again, the turnover of nitrogen 15 is what you're looking at as the turnover of proteins. So the inclusions are right, but the nitrogen may not just be an incorporation of lucine into proteins, and then taking it out. It might have a longer retention because it stays in the different amino acids a longer time. I want to get around that. And we'll come back to that in a few minutes.

Go on to this oxygen labels (Slide #26). Again, it comes from the cryogenic distillation of NO, in our case at least (Slide #27). So we're making everything, say, from NO. And it's easy to get O18 water, or O2. Those are the kind of molecules we start all our labeling chemistry from. This is a common approach to make hydroxol groups label. So first of all, we make an O18 reagent, which is benzoic acid, which has O18 in both positions by hydrolizing this (trichloral) molecule, this trichloride. And I just want to point out that when you do this, you're using a (limiting) amount of O18. So if you go to do this in your lab yourself be careful because you're actually making HCL gas. And I can tell you from experience you generate a lot of pressure when you're doing this reaction. So you have to be very careful with this.

Then (Mitzenovo) described these conditions – (trifenelphosphine), and (diethylazodykerboxolate) – that activate hydroxel group, and turn it into a (leading) group. So what we do is generate an ester where this oxygen leaves, and that incorporates O18 into that position. Then we hydrolyze it, and we're left with an O18 hydroxel group. And you can use this, for example, we'll show you that you can label sugars this way quite readily.

And if you're clever about it, you saponify with O18 sodium hydroxide. And that way you regenerate the reagent that you started with. So you can go on and carry it through more cycles of this, rather than just having the label (wash out).

Okay. There's also a problem with oxygen 18. And this will come up with some of the labeling that we're trying to do to look at lipid turnover. So O18 is stable if it's in a hydroxel group, but as soon as that gets oxidized, formaldehyde or a ketone, it can react with water to make this (dyal) (Slide #28). And the effect of water washing in, and then reformation of the aldahyde or ketone is the loss of O18 to the water. So you have to be careful that when you're trying to label it and retain it in a position that it doesn't go through an aldahyde as a metabolic intermediate.

Okay. So finally, C13, which has historically been my favorite isotope, has one problem from MIMS, and that's the (high) background (Slide #29). And we'll talk a little bit about how to get around that. Separated by cryogenic distillation of NO, high background (Slide #30). What that means is we think we have to label with multiple carbons. So you increase the signal.

One problem, it is challenging chemistry to make molecules as complicated as biochemical molecules sort of one carbon at a time, making all the carbon-carbon bonds.

The one thing that's done in industrial that's quite useful for compounds that are labeled for MIMS or for these methods is that they oxidize a CO to CO2, and then they grow algae, and they isolate compounds. If you look around, there's lots of uniform label compounds in (catalogs), and this is where it's coming from. The good thing about it is the labels stay where you put them in the carbon skeletons. And actually, many of the metabolic questions actually are carbon-related.

So the chemistry's hard. (Again), you make everything as separated as CO. You have to make the precursors. Historically we reduce CO to methanol, made that into methylide. I showed that before. Can easily make (formate) by reacting CO with sodium hydroxide. Cyanide is two steps. You make methane, and then convert it to cyanide.

And the most useful forms here were cyanide and methyl iodide, so you could alkalate things to make carbon-carbon bonds, or you could displace halites with cyanide. This would leave you with methyl group, which is hard to do chemistry on. This leaves you with a (carboxolate), which you can do chemistry on, but you usually have to adjust it. Again, CO2 is good for (grenyard) kind of reactions. So labeling carboxolates (was) straightforward.

What we always lacked was sort of the oxidation state that you need, which is formaldehyde. There's lots of literature on how to make formaldehyde. None of them work very well. And it has to do with the instability of the formaldehyde itself. So we saw ways to get around that problem, and it sort of came from the chemistry I'm talking to you about. We made this (thioanosol) I talked about earlier. And this has got the advantage that you can do lots of chemistry with this methyl group, and then cleave off the sulfur in a lot of different ways to either leave you with H, or O, or anything there. And there's all these kind of precursors that you can talk about (Slide #32). Here's a (mask) formaldehyde, for example, just at the right oxidation state to do most of the biology. We're exploring the use of this, and it's shortening,

actually, a lot of the routes to our label compounds. And I'll just show you – well, this is kind of to show you how the carbon flows from CO to methanol. Then we make thioanosol straightforward, and very high yields, all of these steps. And thioanosol, like I said, it can be activated so it's either a (nucleophile), and you can add the halite there, or it can be activated so it's sort of an AC/DC molecule. It can either be electrophylic, or nucleophylic. And you can either then add it to things, or add things to it, and make intermediates. And the nice thing is we've developed (facile) methods for adjusting the oxidation states, so you can make that into a carboxolate (analdehyde), and you can go on and do more chemistry. You can make it into a methyl group. So these are sort of the advantages of trying to use this molecule. And it shows up in the synthesis of (ethyldiamethyloximate). And this seems like a simple compound, but this you can go on and make into alphaketo acids, and amino acids readily. But it was never available as a labeled precursor.

And what we've done here is actually introduced labels so we can differentiate both ends of these molecules. You can make it one C13, or two C13, or double labeled. All of these syntheses (weren't through) symmetrical molecules. But (dioanosol), we add CO2 to that. Straightforward. Generate the acid chloride, and add the amine. Then you can – this is the way we oxidize it. Just make the dichloro, and hydrolyze it. If you hydrolyze in the presence of ethanol, you get the ethyl acid, ethyl derivative.

And this just shows you can use that to make – we wanted to make glycerol, but again, this is a symmetrical molecule that you make asymmetric by adding labels to it. So we generate this stereo chemically pure from the diethyloximate. We added this (kyral) molecule. You get the three carbon backbone actually here, and the reduction is directed by this (kyralsulphoxide to) only come from one side, so you reduce that molecule. Then you just have to (deblack) it to get the glycerol.

And again, we checked the stereo chemistry here by using glycerol (kinase), which can differentiate the (Ns) of glycerol. It will only put on phosphate, so you generate L glycerol (from) 3 phosphate. So if you start with the labels in two of the carbons, you'll wind up with one two C13. Or if it's in the other carbon, it will wind up as two three.

This is the carbon 13 spectrum of this molecule. It's complicated because it's coupled to phosphorus. See those small couplings. Coupled to each of the carbons. But the ratio of the labels at one and three show you that we have about 90 percent stereo chemical purity, in this sample at least.

You want this – again, this is for NMR, but it will be used to label proteins as a carbon source for growing ecoli, and expressing proteins. And if you do this with the right kind of labeling pattern, you wind up with amino acids that are – at least these amino acids are all labeled this way, with one C13 at the backbone positions, and then deuterated in the (methylene) position. This is sort of ideal for making assignments in large proteins.

And all you have left are proteins from two families. The (alophatics): valine, lucine, and isolucine. And then those that come from glutamate: glutamate, glutamine, argenine and proline. So you could add, say, label glutamate that had this kind of chemistry, and valine and lucine, and you'll get all the amino acids incorporated this way. Yes?

Andrew Davis: Can I ask a question?

CU: Yes.

AD: In that case, the deuterated ?? (is going to) wash out ??.

CU: No. And also in this case we grow in the presence of D2O, so if it washes out, it will be – it's retained in almost all of these. There may be one or two amino acids where it washes out, like alanine itself.

Okay. So now I wanted to introduce this concept of correlated labeling (Slide #39). And this is what Claude was referring to over here. And this was actually developed in the late seventies, and (Seto), and then Gould and Kane about the same time had two papers. And what this did was it changed natural products chemistry from being carried out, say, by (Floss), and Baldwin, and (Cory), and all these brilliant chemists who could look at a molecule, and predict how it was going to be biosynthesized, and make a precursor, and show that it would come in, to where people like I could do natural products chemistry.

And what they did was they showed that if you introduced multiple labels, you could put a correlation between these labels. This required, in their hands, NMR analysis, but I think there's MIMS ways to do this too. So the idea is if you, say, have a mixture of a uniform label compound, say at 10 percent glycerol, then 90 percent of an unlabeled compound, when you went through biosynthetic steps, those bonds that were retained from glycerol will have a high correlation. So if one of the carbons is labeled, then 99 percent of the next carbon will be labeled, and 99 percent of the next carbon will be labeled. However, if it's a new chemical bond that gets formed, then the probability that the neighbor's labeled drops to 90 percent.

This idea of multiple labels, and this correlation in the labels has sort of led to a revolution in natural products chemistry. And you can see that this is what happens (as) you analyze your molecule. Most of it will be unlabeled. All the carbons will be labeled at 10 percent, or 9 percent of the molecules. Glucose will be labeled at one, two, and three. (9 percent) at four, five, and six. And then only a small amount labeled in all the positions. And what this let you do is establish that, because when you found C13 at one, there was a high probability it would be at two and three, but then a low probability it would be at four. If you found it at six, it would be a high probability at five and four. So this let you get at labeling (compounds).

Claude Lechene: Cliff, the total is (101) percent.

CU: Oh, okay. Yes, I'm sorry. It was late last night. Late last night. I'm sorry. So anyway, like I said, this let people like me do natural products chemistry. So we worked a long time ago on the biosynthesis of this molecule, which is (pyroloquino and quinone) (Slide #40). It's a coenzyme made in methylotrophic bacteria used to oxidize methanol. And we had showed earlier that, from some labeling experiments that, say, (tyrosine) was probably a precursor for this part of the molecule. What we wanted to do was establish (that) tyrosine was incorporated as a complete molecule, and it wasn't broken down. And this sort of illustrates how I think we can use this with MIMS. So again, we did NMR analysis (Slide #41).

But what we did was we (synthesized) tyrosine that had two carbon labels, and in the same molecule an N15 label. So what happened was (then it) got incorporated, you made this bond, and you then had a direct N15, two C13 molecule. Also, because these two carbons could be coupled in an NMR experiment, we could see that this part got incorporated intact as well, because again, wherever there was a C13 here, there was a C13 in that position. And I just showed the analysis of this one carbon. So what happens is that it's a (doublet) again, because it's C13 labeled (Slide #42). Then when you form this bond you get N15 labeling, which is one bond coupling, so it's bigger. So you get (a) ?? split like that.

In reality, what happens is that there is transamanation like I talked about before, so you dilute this label some. So you get a mixture, actually, of the N15 labeled stuff, and that that has N14 at that position. And this is old, so ??. But this is just looking at the C13 at that one position. And you can see we saw the retention, actually, of 100 times more N15 than you would predict if N15 had equilibrated with all the nitrogen (pool, in the) ??. So we took this as evidence that tyrosine gets incorporated completely as a precursor.

So how can you do this in MIMS? Well, one way is to make molecules that have correlated labels. And this is an experiment that isn't finished because I didn't get all the synthesis quite done. But Claude's – this is his idea to try to start to understand the cyanide production. If secondary (ions) are formed where there was originally carbon nitrogen bonds at a higher rate, then we'll be able to do these correlated type labeling experiments.

So we've made these five peptides (Slide #43). (They'll be in) HPLC next week, and Claude will have them next week. So he can start to generate – start to understand the cyanide production from the peptide backbone. Again, this is (the simplest) glycine amino acid backbone that we can make. And we've put the labels either everywhere, with uniform labeling, or we've put them one bond apart, or we've put them next to each other, like they would form when you make a peptide bond. Or next to each other like they would be if you labeled alpha carbon and the amino group. So we'll understand then the cyanide ion production from that molecule.

We're making these molecules, again, so that we can hopefully correlate cyanide ions and nucleotides. Again, where the bond gets retained, perhaps, in the cyanide ion, or whether the probability is the same if it's just in the same molecule or not (Slide #44). These things are a little bit out of order here. Okay.

So this is the experiment Claude was talking about where, again, you'd introduce a correlational label in (ribo) nucleotides. So the idea is to put O18 at the two prime position, and then N15 in the (site at the) – or in the base, in the purine ring molecule. And, of course, C gets incorporated into both DNA and RNA, and ribonucleotide reductase is the enzyme that converts the – in humans, actually, it's the diphosphates, I guess, to the dioxynucleotide, and this gets lost to water. Okay? This will get pumped away. So the idea is that we can sort of watch at least the metabolism of nucleotides to dioxynucleotides. We know where to look for the products: either in RNA or DNA. And again, lose this correlation, the ratio of O18 to N15 should be greatly altered in dioxynucleotides compared to ribonucleotides.

You can do the same thing in protein turnover (Slide #45). So the idea here would be to put two O18 labels in an amino acid. And then you could either correlate that to a C13 label, or

more likely, an N15 label. Put them all in the same molecule.

So when you synthesize proteins what happens is you make an (amid) bond, and you lose one of these oxygens. Then when you rehydrolyze it, it gets hydrolyzed from water. So it will be back incorporated as O16 O2. The idea is that what you feed has this O18 to C13 or N15 ratio of 2. After you make it into a protein, when it's in the protein for the first time, it has a ratio of 1. And then as you hydrolyze that with H2O, you get carboxolates (that) ?? (one). And as it goes back through the cycle again, you'll start to have a mixture of 50 percent O18 labels, and 50 percent O16 labeled at this position. So as the amino acid gets hydrolyzed out of proteins and reincorporated, this ratio will drop eventually to zero. That's an idea about how you could look at protein turnover in maybe a more quantitative way, or at least use this to calibrate the N15 experiment by doing this one time, or something.

This is synthesis of the O18 (Slide #46), which is still at this point where we've made the O18 labeled sugar now. So what you can do is exchange O18 into the C1 position, which – the picture this should be O18 here, not here. So O18 gets incorporated because essentially this is an aldahyde. So in a long period of time it will equilibrate with water. So we've incorporated O18 into that position.

There's this (malibdate) exchange reaction (Slide #47), described by Barker a few years ago, where when you treat sugars with malibdate, you actually get a carbon skeleton rearrangement, and the oxygen gets carried along with it. And also you (epimerize) this position. So what happens is O18 moves from here in aravonose, to here in ribose. And that will trap the O18 in the position we want it in. We've made this amino acid, it gets all blocked out as (fenswheel) groups. And we have the cytadine now made, and we're going to hook these together, and give this to Claude to study that reaction.

I guess the other thing to get around the C13 is to incorporate multiple labels in things. Like I pointed out before, there are – we haven't done a lot of lipid chemistry, but there's many lipids that are available that get made by algae. The ones that are there in high concentrations are already available commercially. But there's lots of other lipids that are more important when you start doing the kind of cells that, you know, human cells, and (yucuriotic) cells (Slide #48). And we propose to make these not uniform labeled all the way, but to make this a precursor that could be added to a bunch of (side chains) that would let you get to lots of different amino acids. So there's two routes to make either the (more) normal straight chain fatty acids; or (arachadonate) through this procedure. That will let us put four or five C13s at the carboxyl terminal of those. Shows the Nicolauo procedure for making arachadotic acid. Again, incorporating our C13 label molecule (Slide #49).

And then the last idea was also this – we're working on this with Professor Kleinfeld and Claude. And they've done the first labeling experiments with the lipid. But we're also trying to incorporate O18 here. So again, when you make triglycerides, and hydrolyze them, O18 goes away (Slide #50). If you have the O18 in this carboxolate, or it gets incorporated if you have the O18 in glycerol.

We're making O18 labeled glucose, actually, which is the only way to get glycerol into his adiposites. And then we're also taking the C13 labeled amino acids and exchanging them

with O18. So we can watch, relative to the carbon label, either the wash out of the O18, or the glycerol incorporation of O18.

And this was a case where you have to be really careful where you choose to label glucose. Glucose is a precursor to making glycerol 3 phosphate. And we started looking at this. Where can you – you know, there's six oxygens. Where can you incorporate it that you know it will stay in glycerol? And because of this exchange problem with aldahydes, that eliminate immediately the one position, because it will exchange back out if you (draw in the) straight chain (form, that's) analdahyde. When you convert that, in the first step of metabolism, to fructose, C2 becomes analdahyde, so it will exchange in that position.

When you use (aldalase) to cleave fructose 16 (bisphosphate), you get these two trioses: the hydroxy acetone phosphate, and glyceraldahyde 3 phosphate (Slide #51). And those two equilibrate. So that eliminated two more positions that were aldahydes: what came from C2 of glucose, and C4 of glucose. And you're left with the only possibility of labeling it C6. So if you incorporate O18 into C6 of glucose it gets retained by this (phosphorilation), and it gets retained all the way in glycerol. There's no chance for it to exchange out.

So again, this synthesis is actually done. This is the one I referred to earlier. We made the benzoic acid that's labeled, and we used that to incorporate into C6 of glucose, an appropriately black glucose molecule (Slide #52). You hydrolyze it. You have O18 there. And then you crystallize it, and send it here. So that's where we are right now with that.

Okay. Just before we quit I want to tell you about the rest of the researchers in stable isotopes resource (Slide #53). The primary organic chemists were Pete Silks, and Rudi Martinez that proposed most of the chemistry that we're talking about here. And just because, for Claude's sake, we've just added four more PhD chemists. (Sig Lodwig), Robert Williams, Phil Stotter, and (Lillian) Wu. So we'll start to get compounds out a lot faster.

CL: I hope.

CU: Two post-docs, and these are technicians. And MaryAnn Martinez is a resource administrator. Thank them, and thank you for listening. [Applause] Yes?

William Lamberti: So what's wrong with C14?

CU: Just that – I mean, C14 is – to get it at a high amount, you have to have a lot of radioactivity. Okay? So if you're using it as a tracer, you have a very tiny amount of label that's in there. And so what's wrong with C14 is that it's a beta emitter, and nobody wants to use it in their labs anymore; at least not in the US.

XX: ?? radiation (is a real) problem.

CU: Right.

CL: What's wrong is the amount of paperwork to use it. That's basically what's wrong. (It's not) ??.

XX: But the background is nothing.

CU: The background's nothing, yes. I guess one thing I forgot to say. I mean, the background – it's another sort of advantage that we have to figure out how to take care (of it). We talked some this week about detecting multiple labeled species, multiple C13 species. And the reason is that you can also lower the background by a factor of 100 every time you add a C13 label, right? So 1 percent of the natural abundance of C13, but it's .01 percent for double-labeled species.

So that's another strategy that's been used, actually, in natural products chemistry is to lower the background by looking for these multiple-labeled species. (They just) are only there in a vanishing amount in natural abundance. Any other question? Yes?

WL: Well, first of all, it's a great resource. I'm very impressed with the breadth of compounds that you can produce. And I know you've sort of emphasized the biological end here, but if we wanted to approach you to produce certain compounds, say, what's the procedure, say, for an industrial, or a partner that's not in collaboration with you at this point?

CU: Right. So we have a website, which is Sir.Lanl.gov. And you can approach us through that. Now the one thing I would say is that we – I mean, we have lots of intermediates that can easily lead to, say, polymers, and things like that. And there's actually quite a lot of interest in that amongst the chemists that are there. They all didn't start doing biochemistry.

So those kind of compounds that we have that are close, or that you can do chemistry with, we'd be available. But really our funding source is NIH, so there's two things that they kind of require of us. One is that it's really biomedical applications that we're pushing. Now we still have done – we've done astronomy, and we've done all kinds of things. NIH is also just interested in good science too. So there's possibilities to do that.

The other thing is that we like to have – the main product we get is acknowledgement of our grant on your papers. So I'd be most interested in things where it will eventually, even if it's a long ways in the future, lead to publications. And that's important to us. And then the other possibility is if those are – if we do sign, we have signed (funds in) agreements, and confidentiality agreements with private industry too.

WL: Okay. And who would be another – what other types of groups that are out there producing similar compounds?

CU: Well, there's three or four commercial outlets in this country that are Cambridge Isotopes, and Isotech. And then there's a new one called (Spectra Stable Isotopes). And actually with those guys I can say that we have an agreement with them, so we're doing – we're actually renting the separation facility now to Spectra Stable Isotope. So they're actually producing isotopes at Los Alamos. And they grow algae, so they have a lot of these algae-growing things.

But I do refer everybody to – and our web page does to private industry too, because if it's commercially available, and it's not – and you can at a price where you can actually do your experiment, that's their job. Our job is actually developing new things. So we don't have – you know, I don't put – like our idea is to make these labeled valines, get them into the literature, and

then hopefully have it taken over by private industry.

I think what attracts us personally to projects right now is these multiple-labeled kind of things, because that's not being approached at all by private industry. You can find a mixture of N15 and C13, but again, it's just uniform-labeled molecules. But for the most part they're focused on single C13s, or these compounds you can identify uniformly. But you don't see a combination of deuterium, and C13, and nitrogen, and oxygen in the things they sell. Yes?

HV: How about incorporation of labeled isotopes in inorganic (phases), for example, polymers, or minerals, or carbons?

CU: Polymers are not inorganic, are they?

HV: No. I mean, nonbiological, let's say.

CU: Right. Well, like I was telling him, we have precursors – many of the compounds that we've made as precursors for biology (can) polymerize. So there are those molecules around that we've made, but actually no one else has. So you should contact us. Like I said, you know, I have an advisory committee. If they say it's an important science problem, then we go ahead with it. We try to – we do have this NIH focus. They're the guys that fund us, and we're mostly interested in funding people that have – I mean, the best thing is that they have grants from the NIH, or some – but they don't really care that much about that. Claude can tell you. They care mostly that it's published in open literature. And really it requires you reference our grants. Yes?

AD: Maybe we can talk about this afterwards. But I was interested in sulphur. You're not talking about sulphur, and sulphur's not widely available. Maybe this is a discussion for afterwards, but.

CU: Yes. Sulphur we've done – we have, actually, purchased sulphur from the DOE, or had the users purchase the sulphur, and then we've converted it into (cystine), and other kinds of molecules. So we have done that kind of chemistry. It turns out that the other – Pete Silks, in particular, is very interested personally in (selenium) chemistry, and he's put – and you can see the emphasis on (bioacid).

So we have put sulphur isotopes in things. They aren't (readily) available to us either, so we have to buy whatever we put in. So that would be a negotiation. The place to start, referenced on our web page, is actually the DOE site. I would go directly to the source for sulphur isotopes. I think especially at a DOE lab you can probably get a better price than you can having Cambridge buy it from them, and sell it back to you.

AD: Are there particular issues with sulphur that you would just mention now?

CU: Well, the ones that are most available are there in high natural abundance. You have to watch that. The chemistry, actually, I guess the one thing, there's not a big background of sulphur. Maybe Claude can comment on this in cells. So you have just cystine and (methianine). They're actually commonly the limited amino acids in proteins. You'll find, many times, one or two methianines, and a few cystines. They're not – you're not going to get

big signals, I don't think; a lot of sulphur itself. But it could answer some real important questions about proteins, I think.

AD: Are there wash out issues?

CU: Not that I – no, not that I can think of. Sulphur's pretty much where you put it.

HV: I have a question ??. If you use a polymer ?? (as a track) ?? reagent. And then it goes into the cell, and we know that it's degraded, and C13 will be available. Do we have any way to see where it's going, and then what (it's going to) –

XX: ??.

HV: Yes, if the C13 is released into cytoplasmic (cytosol), and then bonds to something. Could we use that as a kind of – or it's going somewhere that (we can trace). Because the issue is these polymers are – I mean, they degrade into the cell, and then the C13 I assume is released.

CU: I mean, the ones I know about are polylactic acids, and those will go straight into metabolism, and they'll show up pretty much everywhere, yes.

CL: I am not sure I understood quite the question. The polyglycine –

HV: Don't forget, it's not (voluble). I mean, it's going to be (decomposed) in maybe ??.

CL: Let's try to give a definition of what I was talking about. The polyglycine experiment, this will not be done with cells. These will be experiments which I don't know what will be the (matrix). But the first one I will put a drop of (this stuff), and go in and see what (get) out. And with different mixture of (that) mixture, with part of it, a different molar ratio, which (are not labeled at all) ??.

So this will be absolutely without biological (substrate). So I don't even foresee to put them in cells, except to make (a mash) of cells, and to incorporate it as a (matrix). But even that I am – it will ?? (things).

Now there is another program, which I didn't discuss yesterday, which is when we do all this ??, we assume that we are in steady state. Meaning that input equal output, degradation equal (synthesis). And there are situations, particularly (during lipar). And I believe this is why we see the higher one because we bring more bricks in the house (than) there are bricks that are falling apart. But there are ways, and we are thinking of truly dissecting (both) ??. I am talking metabolic equilibrium, or steady state, or nonsteady state of (any kind). And it's to do double labeling. So, for example, we have begun to do that. You pre-label your protein (two weeks) of label with C13. And at the time of the experiment, you switch from C13 to N15. So now you will have – when you will do your experiments, when you will take your sampling, you will have a decrease in C13, and increase in N15, and you can play games with (those).