

Lab 13A • 06/06/12

The pI is the $[pH]$ at which you have the maximum amount of the neutral form of an amino acid. That pI is kinda like a guidepost, where you have the most neutral. What happens, as we do vary the pH , to the composition of an amino acid in solution? What if we're not talking about just one amino acid; what if we have a mixture of different amino acids that we're trying to separate somehow? It turns out that we can use pH to separate them if we also use charge.

First, let me write down the two example molecules we're going to use in this discussion. Let's start with [I]-aspartic acid. If we're discussing which of the forms of amino acid exist in solution – its anionic, its neutral, its cationic forms – it would make a lot more sense and write the structure of the amino acid as it would really exist in its neutral, cationic, or anionic forms. The top carboxylic acid group has a lower pK_a than the bottom one, so technically, if it's the neutral form that I'm showing, that particular carboxylic group will be deprotonated, and the amino group is going to be protonated. Imagine if we were looking at the molecule as if it still had three hydrogens on it – the protonated hydrogen that's on the amino group, and let's say that we did not yet have deprotonated carboxylic acid groups. In order, the pK_a values: pK_{a1} , which means the carboxylic acid unit, is 2.09; pK_2 , which always refers to the amino group, regardless of side chain, that is 9.82; and then we have the side chain, in this case, that does have the ability to ionize, it has a pK_a of 3.88.

Let's draw the same type of graph we did for a simple amino acid, where we showed the proportion of cationic, anionic, and zwitterionic forms. This graph is going to be a little bit different, cause there's a fourth form of this amino acid. If I started out in purely and completely acidic conditions, you're going to have everything protonated. Take one proton off and it's going to be the one that I've already done here, the more acidic of the two carboxylic acids. As we increase pH , which means we're getting more basic, we're going to increase how much of the second [carboxylic acid] dissociates. Then, the last thing that's going to come off is the hydrogen on the amino group. Let me show you the graph that would be generated; it's going to look much like what we had for the more simple amino acids. Presuming we start at really acid conditions – call it pH 0 – and then we increase pH , that's our x -axis; imagine our y -axis is either mole percent or mole fraction. We'll start out with [everything protonated, with it] in its acidic or cationic form if you're at low pH . Then, as we allow that first proton to come off, then we'll end up with the neutral or zwitterionic form. As it loses a proton, it becomes neutral; at some point, the maximum amount of the compound turns into its neutral form, then yet another proton comes off, which would be the second of the carboxylic acids. If that starts to come off, then instead of it just being simply neutral, it's going to be anionic. But this has the ability for one more dissociation. If every that can dissociate does dissociate in this molecule, we're going to have two negative charges – because of the carboxylic acids – and then the amino group will become neutral, so this is going to become a double anion. Notice the various crossover points on this graph. The first place – where we'd have the same proportion of the cationic and neutral forms – would correspond to $[pH]$ 2.09; the point where we have the maximum amount of the neutral form – that is the pI , which for this compound is 2.98; then we have the place where we'd have the same amount of the neutral and anionic forms, which would be at pH 3.88; then we have where then anionic and doubly anionic forms are equal, that would be at pH 9.82. When the pH is equal to the pK_a value of a functional group, then when you're at that pH , half of that functional group will be in its protonated form, half of that functional group will be in its deprotonated form.

Then comes the question: what do we have in solution at pH 4, for example? Here, what I'm trying to say is that between pH 0 and 2.09, the cationic form predominates. Between 2.09 and 2.98, we have the neutral predominates, but we [also] have cationic, we're going towards neutral but there's still some of that cationic form. On the other side of the pI – the pI is the point at which you have the maximum neutral form – that means you're going to start going towards anionic, so this will be neutral but more anionic. Once you pass that next threshold – which is pK_{a3} , the pK_a of the side chain – then we're in the anionic form. Then, raise the pH enough, and you'll be in the double anionic form. To highlight, 2.09 is pK_{a1} ; 2.98 is the pI ; 3.88 is pK_{a3} ; and 9.82 is pK_{a2} . The pI is pK_{a1} and pK_{a3} averaged; from the graph we can tell that's because that's where we have the most amount of the neutral form – it's because we have two carboxylic acids and we don't wait for both of them to deprotonate, because otherwise we wouldn't be neutral, we'd be anionic.

Now if I ask again what do we have at pH 4? You're just the the righthand side of pK_{a3} , which means we're going to be predominantly in the anionic form. The point I'm trying to lead up to is: how could we separate amino acids using this behavior.

Let's switch to another example: [I]-alanine. For alanine, the pK_{a1} is 2.34; pK_{a2} is 9.69. Since this is the more normal case of having just the two pK_a values, you just average them, which for this compound ends up being 6.02. Let me do the same style of written graph using the same pieces of information. The three pK_a values I have are the pK_{a1} , pK_{a2} , and the pI , so that's at 2.34, 6.02, and at 9.69 [no in order]. Below $pH = 2.34$, we'll have the acidic or cationic form; between 2.34 and pI , 6.02, we're neutral, but with a little bit more of the cationic form; and then above that pI point, we'll still be more neutral but with a bias towards the anionic form; and then at a high enough pH , we switch fully enough over to the anionic form. On this one, we could say: at $pH = 4$, we have more neutral and anionic form are present.

Now, what if we had a solution of both amino acids, and we put this into a buffer solution that had pH 4? The aspartic acid is going to be more in its anionic form, and although more of it's neutral, there'll be a bias of the l-alanine towards the cationic form. Now imagine that we have a plate, a piece of paper, whatever the material might happen to be in real life – across which we put an electric current. Let's say that we start with this mixture of aspartic acid and alanine. Flip the switch on. At pH 4 we have more of the cationic form of [alanine]; cation – plus charge; plus charge – attracted to minus charge, which means the [alanine] is going to migrate towards the negative terminal; that's because the larger proportion of it exists in the cationic form. Then, separately, the [aspartic acid], which is neutral but slightly geared towards the anionic side, since it is a negative charge, that means it would prefer to move towards the positive terminal. This process is known as electrophoresis.

For ninhydrin, in the reaction with an amino acid, you lose the identity of the amino acid, because the R group gets cleaved off during those imine formations and hydrolyses. That's why we use it here, though, because the identity doesn't depend on using ninhydrin; it depends on where does it end up here on this electrophoresis plate. Ninhydrin can be used as a visualization agent, to see the amino acid, but it's not used as a identifying agent because all amino acids make the same thing out of ninhydrin.

Ion-exchange chromatography

Ion-exchange chromatography involves a resin. The example resin that [we'll examine] is very similar to the Merrifield resin, in that it is a derivitized polystyrene. For the Merrifield resin, we had some p-chloromethylstyrene – in other words, a benzyl group. Here, instead, let's imagine that we have a sulfonic acid group. We can co-polymerize the two compounds [benzene and the sulfonic derivative], and then subject it to base to deprotonate all of these acid groups. We'll get a polymer which parts of it will have just regular benzene substituted; occasionally, we'll have a sulfonate salt. I'll call it neutral – it's not because of its charge, it's because of the fact that a sulfonic acid is a really, really strong acid, likes to lose its proton. If we force deprotonation of it, then the salt that forms doesn't want to go back to being the acid. That means that the salt is not basic, it's unreactive – it's neutral.

Let's say we took this material and we pass a solution of amino acids through it. If we have an amino acid that happened to be at a low enough pH where it predominated in its cationic form – there'll be sodium ions all up and down this material, but, temporarily at least, the amino acid can displace a sodium ion. That's the ion exchange, right there. Eventually if a solvent passes through, it could be forced to continue down through the material. Some amino acids may be more in their cationic form than others. Those that are more in the cationic form would be likely to linger on the column because of this charge interaction; those that might be in their anionic form might fly through because they're repelled by the column by the material. That's this ion-exchange chromatography.

I want to talk about what column chromatography is, the preparation and the process. [polymerization initiator – would a strong lewis acid be used for such a functionalized benzene?] Here's the physical set-up of a column. Depending on the scale and, for column chromatography in general, depending on the application, this column might be the size of a regular old pipette, or it might be the size of a grain silo, some huge kind of column. This column is going to be doing separation based on physical interaction, so we want the column material itself to be uniform and level, because if it's not level at the bottom, then whatever solvent's coming through might come off at different times; as we're going to see in a moment, it's the time it takes for something to pass through the column – that's known as the elution or retention time – we're going to use that to identify compounds. To make sure that this column is flat, the bottom of the column is often filled with sand or some other kind of inert material. You'll then fill the rest of the column with this resin. Then you'll take what I call an amino acid soup – if we had some polypeptide chain, if we were to subject it to strong acid, aqueous conditions, [at] an elevated temperature for a long period of time, we're going to chop this up into individual amino acids. If I had amino acids with these mythical labels A, B, and C in a polypeptide, after this treatment, then we'd have all of the amino acids separated out again. The important point here is that hydrolysis means that any form of sequencing information is totally and permanently lost. We could use a particular reagent to pull one amino acid off at a time; this is the brute force approach, where we separate everything at once. That means we won't be able to get sequence information, but we will be able to get how many amino acids there are and of what type.

Coming back to the column, we place the hydrolyzed amino acids (the "soup") on the top of the column, then, generally, we force a solvent through it, because gravity alone is not usually sufficient to pull this amount of solvent that gets used through the amount of material that's used to separate. This is what the column looks like at the beginning; as time progresses, based on pH, different amino acids will take longer to pass through the column. Just to represent this somehow, I'll put two different bands on here and I'll call them "eluting compounds" – eluting means it's coming through the column, eventually coming off. Amino acids are not visible, necessarily [on a column]. But, this protocol, this technique has been done so many times that there's an established way to make the columns – if you follow that procedure and use the same type of material, the same kind of solvent, then you can reproduce the experiment and the the different kinds of amino acid to come off at different times, but reproducibly. So if you know how long it took for a compound to come through the column, you can figure out what the amino acid is.

In ion-exchange chromatography, different amino acids take longer to pass through the column, depending on how much of the amino acid is in its cationic form, and therefore how much it would want to interact with the ion-exchange resin. Amino acids exist in different proportions of their ionic forms, depending on pH. Based on the charge, different amino acids will interact more strongly with an ion exchange resin and therefore take more time to pass through this kind of column. When reproducible conditions are used, the amount of time for a compound to pass through the column – which is the elution time or the retention time – can be used to identify the different amino acids.

If I were to take a graph of elution time versus absorbance, then as different compounds come off the column and we visualize them somehow, then we'll see these different spikes in absorbance. For each one of these different times, it's going to correspond to a different amino acid. I forgot to draw one thing in here: ninhydrin. We would not use ninhydrin until the solution makes it all the way through the column, because ninhydrin destroys the unique identity of any amino acid because you end up making the same complex. But, if we can't see the amino acids, we do want to know that they come off the column, so as the compounds come off the column, then you can expose them to ninhydrin. The reason I've shown you a bunch of beakers like this is because it's somewhat representing how these columns would really be used in real life. You're constantly pushing solvent through, but it's not true that there's going to be amino acids constantly coming off the column. There'll be some period of time right after you start where nothing comes off except solvent, cause it takes a while to get all that compound through the resin. The first one comes off, [but] maybe it won't come off for just a short period of time; these separations are not all or nothing, it's not like: nothing, nothing, nothing, all of the compound in one drop, nothing, nothing, nothing. So, you might have a series of tubes, some of which will have material, some of which won't. Ideally, you want a gap between what are called fractions, which are different portions of an elution solution collected in order to ensure different compounds are kept separate from each other. If the whole point of chromatography is to separate, then you want to collect these fractions in small quantities to ensure that there's some kind of dividing line between the different compounds that may be trying to separate.

As the column is being used, you can sometimes use whole racks of test tubes [describing process, mentioning automated version]. You'd then collect all of the common fractions – everything that came off in one spurt, you could say – and separate out, that would be one amino acid. Because this is all reproducible, you could figure out what time should I start seeing these amino acids coming off. Then, the ninhydrin helps to visualize, to help ensure there's something coming off the column. But, there's one other thing that ninhydrin does. [Beer's law is not: beer before liquor, never been sicker][Candy is dandy, but liquor is quicker] What is Beer's law? [side discussion of Chem 1B spectroscopy experiments] You'd take a particular compound and, as your first pass through, you would vary the wavelength that you observe [with], cause you want to find where the wavelength of maximum absorption was to make sure that you had maximum sensitivity in your measurements. Having established the best wavelength to do an observation, you then would do a series of scans – or sometimes just one scan – the purpose of which is to determine the concentration; that's where Beer's law comes in.

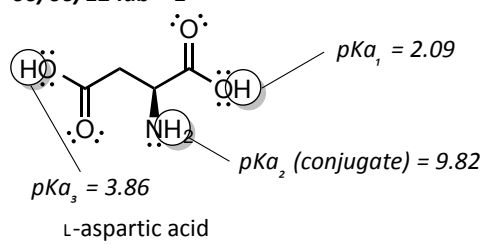
Beer's law is a very simple expression with four variables: A stands for [apple] absorbance – this is all about how does a solute affect light, if you're passing light through a sample, if that sample interacts with the light, some of that light is absorbed – that's absorbance. Turns out that that absorbance is linearly proportion to three things: one of them is path length – imagine that we had a volumetric flask [as used in Chem 1A]. Volumetric flasks are much broader, squat at the bottom and then very narrow at the top. If you take certain kinds of food color solutions and fill these bottles up, you can see the appearance of there being two different colors within the same bottom – once color where the narrow part of the container is, another color where the broader part of the container is. Why? Because light is passing through more of the compound at the bottom, thicker part of the vessel than up at the top. [purple/blue food coloring] The point is: you have these different appearances due to the path length – go through more material, more light is absorbed. Same reasoning can be used to explain why concentration has an effect on absorbance – more stuff, more absorbance. ϵ , or epsilon, that is the extinction coefficient, which depends on wavelength – that's why I mentioned that experiment where you first determine optimal wavelength – but for something like Ruheman's purple, that wavelength's well-established. Then, this epsilon because a fudge factor to relate absorbance with how sensitive is that compound to light. A we can measure; l , we can know that by the way we design our apparatus; and epsilon we know, based on what compound we're looking for, which is Ruheman's purple. If we know these three variables, we can solve for a fourth: concentration.

What if we saved all of the related fractions from a chromatography, for one particular amino acid? We would know what the combined volume of that is. Using spectroscopy, we could come up with the concentration. Concentration times volume is moles. Once we know the different moles of all of the amino acids present, we could come up with the mole fraction, which means know how much of each amino acid was present in the polypeptide chain – which is the point of this chromatography. The concentration of a solution of ninhydrin derivative (Ruheman's purple) can be determined by spectroscopy. Since the volume of the solution can be measured, the total moles of the amino acid that gave rise to that derivative can be determined. This means that the mole fraction of each amino acid in a polypeptide chain can be determined. But, the sequence cannot be determined, because we chewed up all the amino acids in that protein to do the chromatography.

[lab directions][surely you must be serious; yes I am serious, and stop calling me Shirley][guy walks into a bar and says ouch]

Structures (remaining structures identical to lecture 20B)

06/06/12 lab • 1



06/06/12 lab • 2

