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Combined Spectroscopic and Computational Studies of Free and Enzyme-Bound B$_{12}$ Species

By

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7.1. Preface

Working as a teaching assistant (TA) for introductory chemistry courses has really shaped how I think about communicating my research. TAs have a lot of information they need to convey and a limited time to do so. Each course faces certain questions: What are the most important topics to cover in a semester? How can we best prepare students both for their careers and for their lives more generally? Over the past few years, I've found that there are two overall messages that I want to convey. First, chemistry is important. You've probably heard this before, if you've taken a high school chemistry class, but it's worth emphasizing. I've taught courses that covered subjects like climate change, air quality, nuclear energy, ethanol tax credits, and the chemical elements found in smartphones, to name a few. Chemistry is an excellent tool for understanding how our society functions, and learning the basics can go a long way to demystifying topics relevant to our daily lives.

The second message is that chemistry can be fun! My favorite part of teaching chemistry labs is watching students figure out what parts of chemistry they enjoy. Sometimes it's the satisfied feeling of solving a tricky problem, and other times it's a more specific project or technique that we cover. In chemistry, it can be helpful to have a knack for memorization and complicated math, but it's equally valid if you get into the field because you like vivid colors or dry ice or lighting things on fire. I was in the latter camp, and I ended up in a great research group for that. I get to work, for example, with lasers and powerful magnets, and those things never stopped feeling cool—although my childhood self would probably picture something quite different if she heard that I now work with force fields (a computational modeling term!). I hope that this chapter conveys some of the joy I felt doing this research.

I owe many people thanks for the existence of this chapter. First, I want to thank my Aunt Heidi, who helped me discover both the wonders of science and the importance of teaching it well. When I wrote this chapter, I thought a lot about a book she gave me, a blend of history and organic chemistry that delves into some of the molecules that shaped history, from spices and vitamins to antimalarial drugs.[1] It was the first chemistry book I really loved, and it's a great example of how learning a bit of chemistry can teach you a lot about the world. A big thank you also to my parents and sister, who have been a (mostly) willing audience to my chemistry ramblings these last few years. I hope this chapter clarifies some of those ramblings! Finally, I owe an enormous thanks to the Wisconsin Initiative for Science Literacy (WISL) at UW-Madison, and particularly to Professor Bassam Shakhashiri, Elizabeth Reynolds, and Cayce Osborne for their help and guidance as I worked on this chapter. I'm very grateful for the opportunity to learn how to communicate my science more effectively.

7.2. Vitamin B$_{12}$

It might surprise you to know how many different metals can be found in the human body. Some are required in abundance, like the calcium in your bones or the sodium that keeps your muscles and nerves working. Others are required in smaller amounts, such as copper, nickel, iron, and cobalt. Long before early humans started using metals for tools and weapons and jewelry, nature put those metals to work inside living things.

Many of the chemical reactions required to keep your body functioning are facilitated by molecules called enzymes, biological catalysts that speed up chemical reactions without being consumed themselves. Enzymes are built from mass-produced molecular building blocks that are strung together and folded into three-dimensional shapes. The atoms involved are primarily non-metal elements that are found in relative abundance in both the human body and the world around us: carbon, hydrogen, nitrogen, oxygen, and sulfur. However, those elements are not always sufficient to accomplish particularly difficult or unusual reactions, and that is where metals come in. Metalloenzymes incorporate metal atoms into the mix, using the unusual chemical properties of metals to perform different types of reactions. A classic example of a metalloenzyme-catalyzed reaction is nitrogen fixation, through which bacteria convert inert atmospheric nitrogen (N_2) into ammonia (NH_3), a form of nitrogen that other living species can use to build more complicated molecules.[2]

Some metalloenzymes integrate only a single metal atom into their structures, but in other cases, the metal atom is packaged into a larger chemical structure, such as the cobalt-containing molecule known as vitamin B_{12}. Vitamin B_{12} has the distinction of being both immensely complicated and vitally important to human health. The term "pernicious anemia" was coined in the mid-19th century to describe a disease characterized by fatigue, weakness, and a slow decline in general health, usually culminating in serious digestive and neurological symptoms.[3] At that time, the disease was invariably a death sentence. In 1925, Frieda Robscheit-Robbins and George Whipple reported the successful treatment of anemic dogs with cooked liver,[4] kicking off a decades-long quest to isolate the factor producing these effects. His work on dietary treatments of anemia later earned Whipple, along with George Minot and William Murphy, the 1934 Nobel Prize in Physiology and Medicine. We now understand pernicious anemia to arise from problems absorbing or transporting vitamin B_{12}, often as a result of autoimmune inflammation of the stomach.[5]

Whipple, Minot, and Murphy were the first prominent B_{12} scientists to win the Nobel prize, but not the last; Dorothy Hodgkin headed the 1956 discovery of the molecule's chemical structure,[6] using a technique for which she later received a Nobel Prize in Chemistry. This work provided the first blueprint to this essential vitamin, which turned out to be, as the paper described it, "very beautifully composed." For one thing, vitamin B_{12} is quite large for a vitamin! Figure X1 compares the complexity of its structure to the relative simplicity of some of the other vitamins required in your diet. At the molecule's center, a cobalt (Co) atom is enclosed within a mostly flat ring of carbon-carbon and carbon-nitrogen bonds. Chains of atoms strung together trail off the sides of the ring, and one of those side chains loops around the bottom of the ring to bind again to the cobalt atom. Binding to the top of the cobalt atom is a group denoted "R", which simply means that there are different possible structures in that position. While *vitamin* B_{12} refers to one specific structure (specifically, when R is C≡N, a carbon atom triple-bonded to a nitrogen atom), B_{12} more generally refers to the larger family of molecules with different groups in the R position. Notably, for several of the R groups, the molecule contains a bond between cobalt and a carbon atom, a very rare example of a metal-carbon bond in nature!
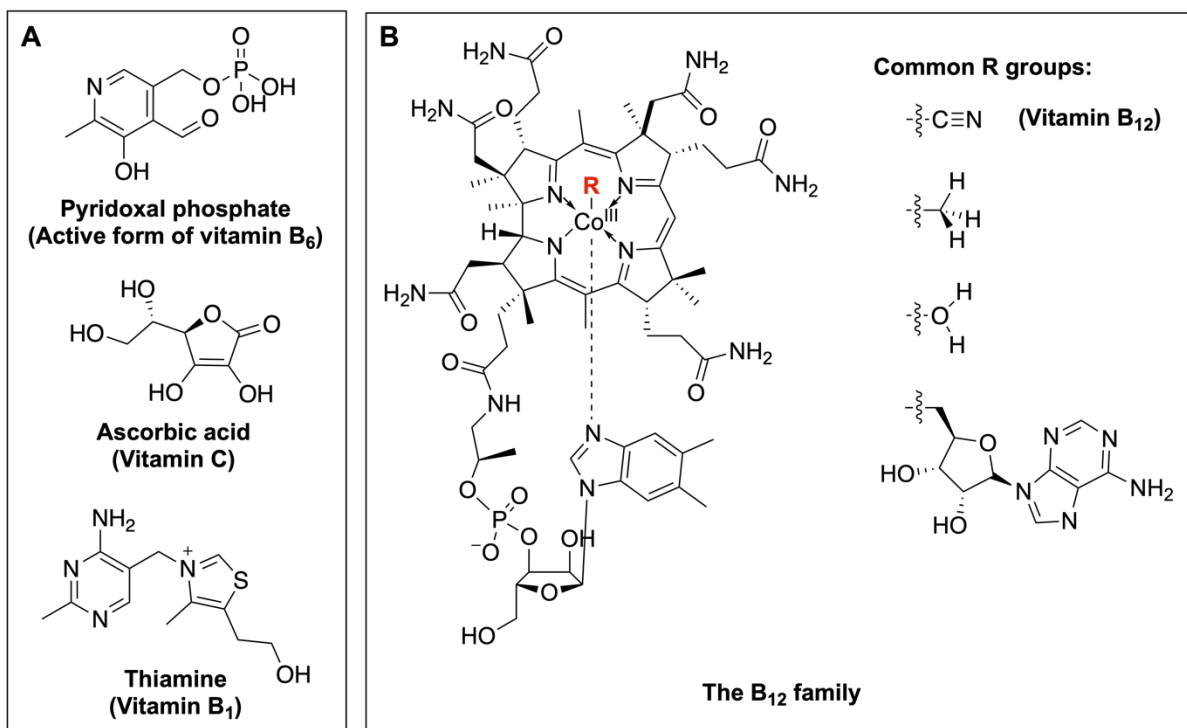
Figure 7.1. The chemical structures of (A) several common vitamins and (B) the B_{12} family. In drawings like these, each line represents a chemical bond, and each vertex or end of a line represents a carbon atom. Hydrogen atoms attached to carbon atoms are omitted. "R" (labeled in red) is used to denote the part of the B_{12} molecule that can have several different structures.

This variability in structure contributes to the B_{12} family's versatility. A true jack of all trades, B_{12} helps enzymes perform many essential chemical reactions for species across all areas of life.[7] Its work can be thought of like this: if a living organism was a factory, then its enzymes would be factory workers and B_{12} would be a tool that they use, useful for a diverse assortment of jobs in all sorts of different factories. Two enzymes in the human body use B_{12} for their work, and impairment of their function has disastrous health effects, including genetic disorders like methylmalonyl aciduria and homocystinuria.[8,9] However, the overall role of the B_{12} family in biology is much broader. Even now, almost a century after Robscheit-Robbins and Whipple published their seminal work, scientists are discovering new facets of the role that vitamin B_{12} holds in the natural world. Recent work, for example, uncovered that a member of the B_{12} family serves as a light sensor in certain bacteria.[10] Chemists are particularly interested in the fact that B_{12} can accomplish such a wide array of fast, controlled, and specialized chemical reactions, which is the sort of skillset that chemists would love to recreate without nature's help.

Much of my research has focused on enzymes that either build B_{12} or use B_{12} to perform chemical reactions. The project described here, however, focuses on vitamin B_{12} itself, and on expanding the tools (both lab-based and computer-based) that scientists can use to understand how it works. This started as a pandemic project, some computational work I could perform at my sparsely decorated basement desk during the early COVID lockdowns, and I had no idea that it

would become such a fascinating and rewarding journey. I hope that this chapter can show you a glimpse of vitamin B_{12} as I see it, as one of nature's most enigmatic and beautiful inventions.

7.3. The building blocks of the universe: atoms and atomic structure

To understand why molecules work the way they do, it is useful to talk briefly about what they are made out of. To that end, there are a handful of chemistry principles worth mentioning here:

1. The most basic, fundamental unit of matter in chemistry is the atom. All matter is built out of atoms.

2. Within an atom, there are three "subatomic" particles: protons, neutrons, and electrons. Protons and neutrons are packed into the tiny, dense core of the atom, called the nucleus, while the electrons travel around them. Although electrons weigh only a minuscule fraction of the mass of the nucleus, the electron cloud takes up most of the space.

3. The specific location of an electron within the electron cloud is fundamentally impossible to know, but probable locations are defined by specific three-dimensional shapes called orbitals. We therefore describe electrons as being "stored" in orbitals, which have specific shapes associated with them.
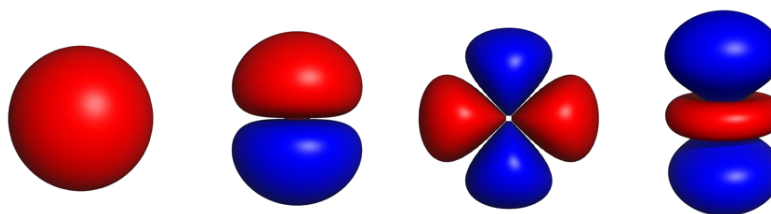
Figure 7.2. Several common types of atomic orbitals.

4. Chemical bonds between atoms are built by the sharing of electrons. When a bond forms, *atomic orbitals* from different atoms combine to form *molecular orbitals*. This means that the size and shape of atomic orbitals define how an atom reacts with other atoms.
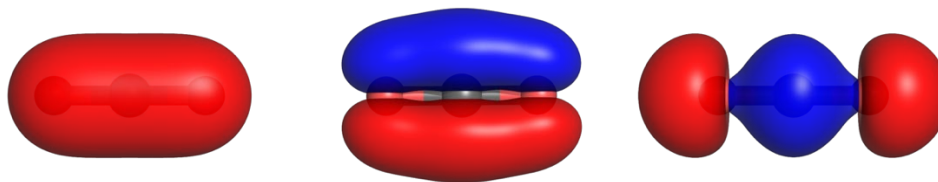
Figure 7.3. Three molecular orbitals of the molecule carbon dioxide (CO_2). The structure of CO_2 is linear, with central carbon double-bonded to two oxygens (O=C=O).

5. Bonds are flexible, albeit to varying degrees. A useful metaphor is to think of them as coiled springs that can be distorted with sufficient force.

6. Molecules have "ground" states that describe the most stable way that atoms and electrons can be positioned, but adding energy can briefly push the system into an "excited" state. This includes electronic excited states (wherein an electron is bumped from a stable molecular orbital into a less stable one) and vibrational excited states (wherein distances and angles between atoms are distorted).

7.4. Spectroscopy: using light as a tool

The arrangement of atoms and electrons defines the way a molecule behaves, but atomic structure is difficult to study directly. I rely on a tool called spectroscopy, which refers to techniques that measure how materials interact with or emit light.

Understanding the full potential of this idea requires expanding the definition of light beyond what is visible to the human eye. First, consider white light, which is a combination of all colors of visible light. This is most readily apparent in phenomena like rainbows and the refraction of light through glass prisms, in which the different speeds at which colors travel through water droplets or glass causes the light to split into its full spectrum of colors. The colors of the rainbow differ in energy, with violet being highest in energy and red being lowest in energy. In this context, energy is not the same thing as intensity. One red laser pointer is lower in *intensity* than one hundred red laser pointers, but an equally intense blue laser pointer is greater in *energy* than the red one.
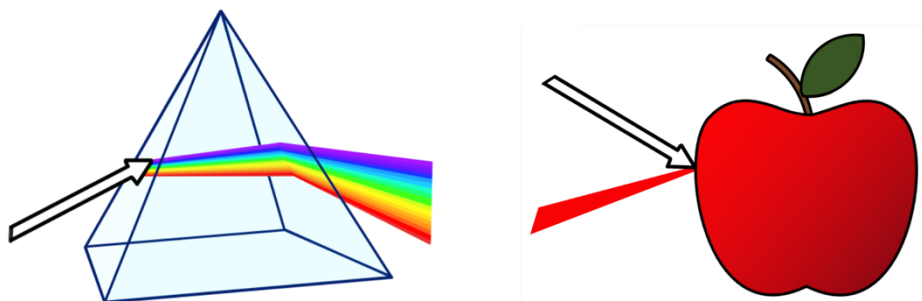

Figure 7.4. Left: White light refracting through a glass prism to produce a rainbow. Right: White light shining on a red apple.

This brings us to the next important fact about light, which is that visible light is one part of a larger whole. Much like blue light is a portion of the visible light spectrum, visible light is a portion of the electromagnetic spectrum. This spectrum covers an enormous range of energies, with innocuous radio waves at one end and Hulk-inducing gamma rays at the other. Different regions of the electromagnetic spectrum interact with matter differently, and these interactions can tell us a lot about different aspects of a molecule's structure or properties. The two forms of spectroscopy used in this project deal with three regions in particular: ultraviolet (UV), visible light, and infrared (IR).

7.5. Experimental and computational investigation of vitamin B$_{12}$

Ultraviolet-visible (UV-Vis) spectroscopy measures how much UV and visible light is absorbed by a sample, and specifically, how much is absorbed for different energies of light. The human eye actually makes a decent attempt at this form of spectroscopy, registering which visible colors are reflected by an object and which colors are not. When white light hits a red apple, for example, all colors are absorbed except for red, and the reflected red light gives the apple its color (Figure X4). UV-Vis spectroscopy uses a similar idea, but with much greater precision and for a large range of energies, expanding into the ultraviolet region.
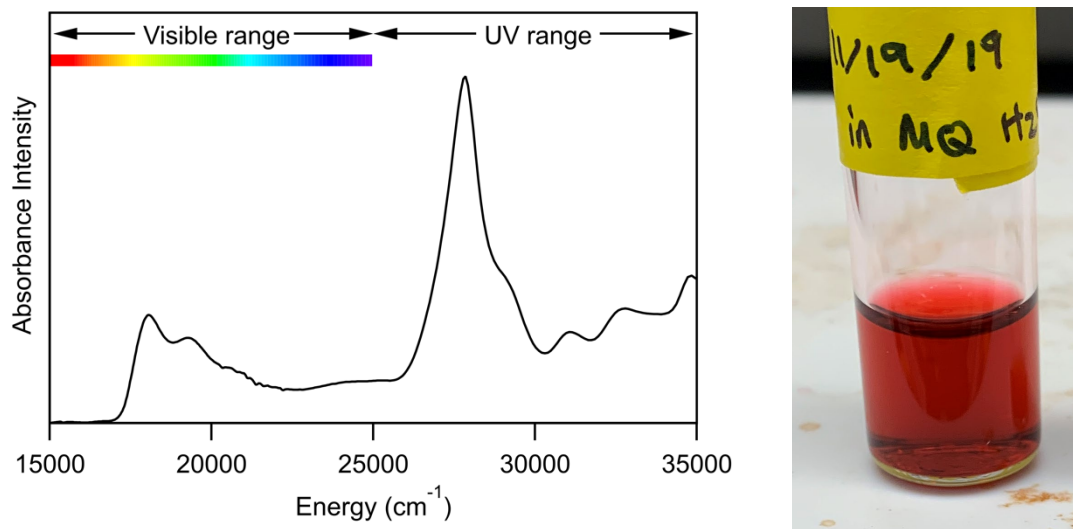

Figure 7.5. Left: UV-Vis spectrum of vitamin B_{12}. Right: Vitamin B_{12} dissolved in water.

Why are only some colors absorbed, and why do we care? Molecules cannot absorb just any amount of energy, only specific amounts that match the energy gaps between the ground state and an excited state. UV and visible light are in the correct range to cause electronic excitations, wherein an electron is briefly bumped from a stable molecular orbital into a less stable one. Measuring the energies of the light that is absorbed provides useful information about the molecule's orbitals, which in turn conveys information about the molecule's bonds and therefore its properties. Consider, for example, the UV-Vis spectrum of vitamin B_{12} (Figure X5). Like a red apple, vitamin B_{12} absorbs red light the least, causing it to appear red in color. Decades of study have demonstrated that the peaks at the low-energy end of the spectrum are very sensitive to changes in the cobalt atom's environment.[11] We can tell, for example, if an enzyme binds B_{12} in a way that changes the length of B_{12}'s Co–C bond or displaces the side chain that binds at the lower face of the ring. This is valuable information to know about an enzyme! To return to the body-as-a-factory metaphor, watching how somebody holds a tool is a great first step to understanding how to use it yourself.

The other forms of spectroscopy used for this project are in another region of the electromagnetic spectrum: the infrared (IR) range. IR radiation is lower in energy than visible light, not enough energy to cause electronic excitations, but enough to cause more vigorous molecular vibrations. In this context, vibrations refer to certain combinations of movements that a molecule can undergo, such as stretching and bending. Once again, the light absorbed needs to have the correct energy to match a specific excitation (in this case, a vibrational motion). Figure X6 shows computer-simulated spectra for two forms of vibrational spectroscopy, IR and Raman

spectroscopies, for the molecule CO_2. Both types of spectroscopy involve the infrared region, but their differing experimental setups cause them to measure different types of vibrational motion. The common convention for IR spectra is to use transmittance of light on the y axis rather than absorbance, which essentially means that the peaks are upside down.

Each peak in Figure X6 is labeled with the vibrational motion associated with it. The peak at 1399 cm^{-1} in the Raman spectrum, for example, corresponds to a symmetric stretching motion of the CO_2 molecule, wherein the C=O bonds lengthen and shorten in unison. The location of that peak (i.e., the energy required to cause a symmetric stretching motion) is dependent on physical attributes like atom mass and bond strength. If you think of a bond as a coiled spring connecting two atoms, bond strength is like the stiffness of the spring; stronger bonds require more force to distort, while weaker bonds can stretch and break more easily. Vibrational spectroscopies like IR and Raman provide a very direct way to determine the strength of a chemical bond—but only if you know which peaks on the spectrum correspond to vibrational motions involving that bond.
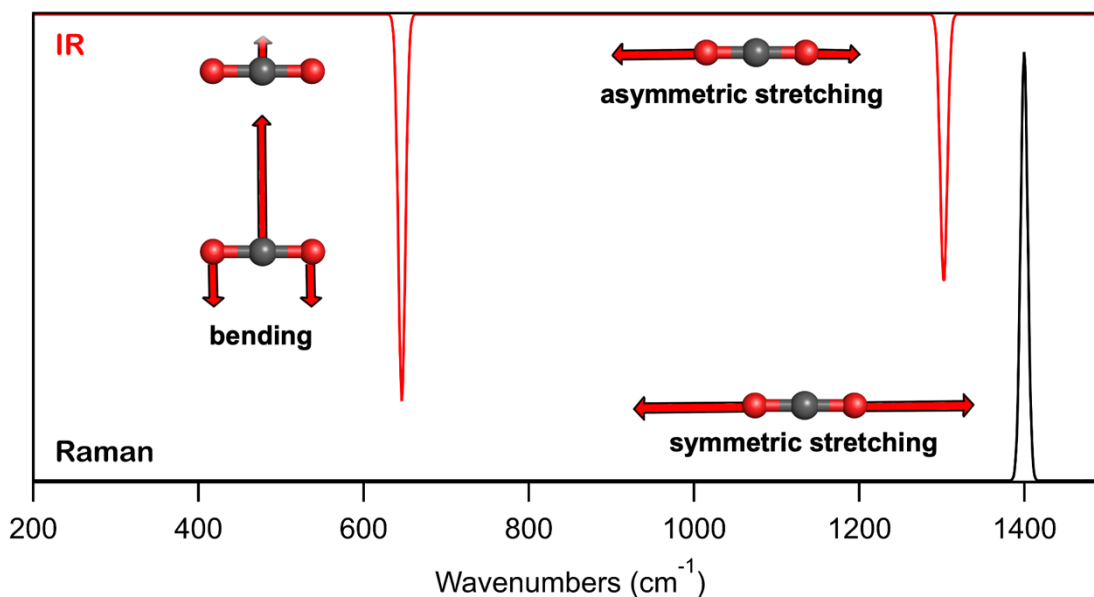

Figure 7.6. Calculated IR (red) and Raman (black) spectra of CO_2, with labeled vector representations of the vibrational mode(s) associated with each feature. CO_2's ability to absorb IR radiation is what makes it such a potent greenhouse gas.

Rather than using traditional Raman spectroscopy, my research uses something called resonance Raman (rRaman) spectroscopy, in which a laser is used to irradiate the sample with a particular color of visible or UV light while the Raman spectrum is being collected. The laser increases the signal for any peaks that involve vibrations of whichever part of the sample is absorbing the light of the laser. In a B_{12}-containing sample, the brightly colored B_{12} molecule absorbs light much more strongly than its surroundings, whether those surroundings are an enzyme that it is bound to or simply the water in which it is dissolved. This is especially useful for experiments in which B_{12} is bound to an enzyme, as it guarantees that the features of the rRaman spectrum arise from B_{12} itself and not from the thousands of other atoms undergoing their own vibrations.
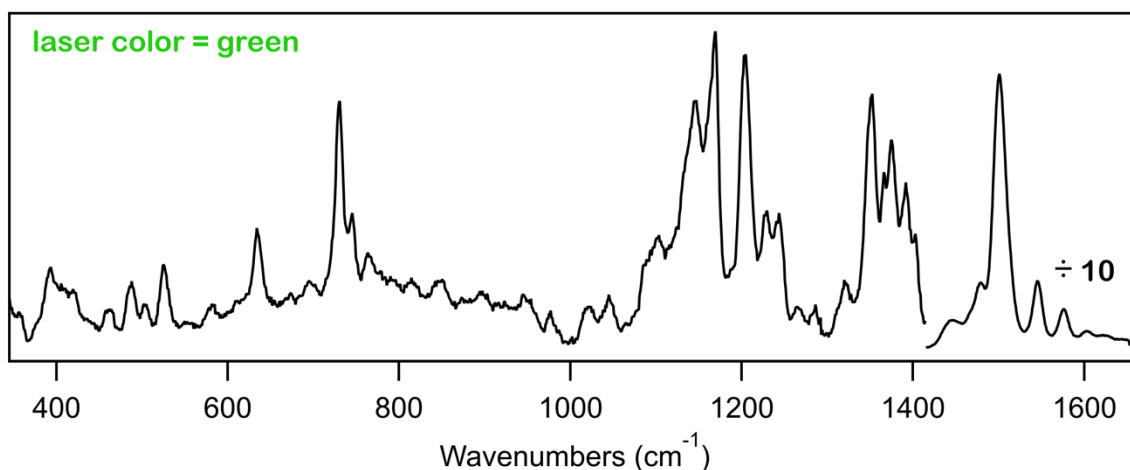
Figure 7.7. rRaman spectrum of vitamin B_{12} obtained using a green laser (energy ~19,400 cm^{-1}). Features above 1400 cm^{-1} are scaled down by a factor of 10 for ease of visualization.

The rRaman spectrum of vitamin B_{12} is incredibly cool to look at, but equally daunting to interpret. Vibrational modes are easier to categorize in a molecule like CO_2, as there are only so many ways a linear, three-atom molecule can vibrate (four ways, to be exact). The number of vibrational modes increases with increasing size, however, and vitamin B_{12} has 181 atoms. Some peaks will overlap, some will be too weak to notice, and all of this with a technique where even humidity can mess with your measurements. How do we figure out what rRaman feature corresponds to a particular vibrational motion?

One way is to swap out one of the atoms of interest for an atom of the same element but with a different mass (called an isotope), which causes rRaman peaks associated with that atom to shift to the left on the spectrum (lower in energy). Subtracting the new spectrum from the original makes it easy to identify any changes that have occurred. Others have used this technique successfully with several vitamin B_{12} derivatives in order to identify Co–C stretching peaks, which are of great interest given that the strength of the Co–C bond is directly relevant to function of those molecules.[12,13] However, until my work, nobody had ever found a feature like that in the rRaman spectrum of vitamin B_{12}.

Another useful tool for interpreting spectra is computer modeling. The equations that describe the shape of an orbital are called "wavefunctions," due to the (probably unintuitive) fact that electrons exhibit wave-like behavior. Equations describing the properties of a molecule are functions that depend on the wavefunctions of its atoms and are therefore functionals (i.e., functions of other functions). These functionals are the basis of a computational technique that is used to predict the properties and reactivity of chemicals. The main challenge is that the math gets complicated *really* quickly. There is no single correct way to approach the trade-off between accuracy and computational cost. As a result, there are hundreds of functionals that have been developed for use in this type of calculation, each with a slightly different approach to the math. Choosing the appropriate functional for a given molecule requires testing and a comparison to experimental data.
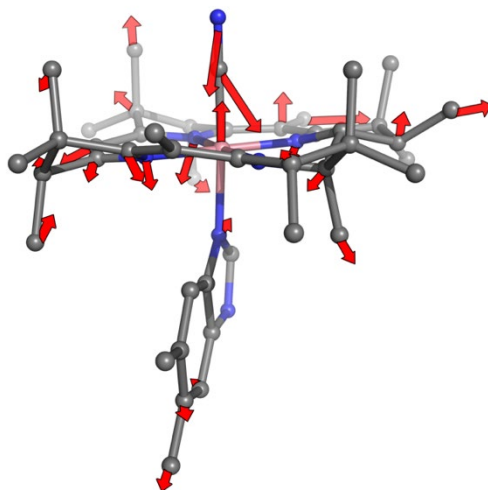
Figure 7.8. Vector representation of a computed rRaman vibration that involves stretching of the Co–C bond and causes a noticeable isotopic shift in the computed rRaman spectrum.

For this project, I evaluated the performance of two popular functionals at simulating UV-Vis and rRaman spectra for B_{12}-containing models. Although the proper choice of functional had been explored extensively,[14–16] earlier analyses were limited by the fact that traditional techniques were able to simulate electronic transitions and vibrational transitions separately, but not together. Modeling aspects like the shape of UV-Vis peaks or the effect of laser enhancement on Raman spectra require that the calculation consider both types of excitations simultaneously. I was able to achieve this using a recently developed software tool,[17] in conjunction with the two different functionals. The differences in their performance will not be discussed here, but there was something equally valuable that I found about what the two functionals had in common: both predicted that I should be able to identify a Co–C stretching peak in the rRaman spectra of vitamin B_{12} that shifts upon isotopic substitution!
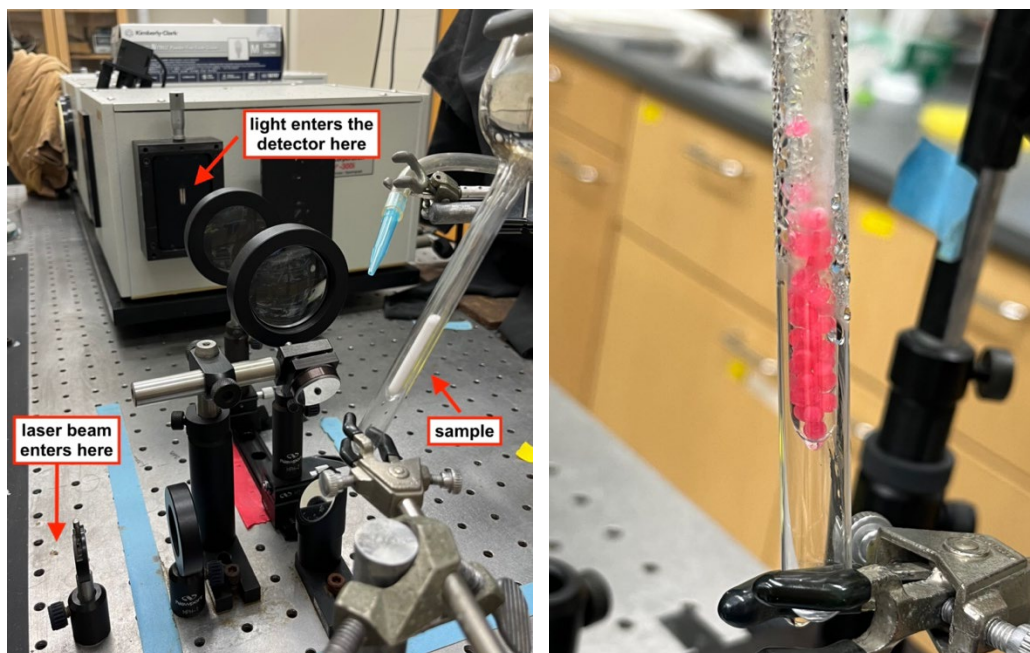
Figure 7.9. Left: experimental rRaman set-up. Right: Vitamin B_{12} pellets in the rRaman sample holder.

So, I gave it a try. Collecting high quality rRaman data is both a science and an art, as it involves very careful adjustments of the experimental set-up. A laser at one end of a table generates a laser beam, which crisscrosses the table through a sequence of mirrors and irises to ensure that a single powerful beam hits the sample that rests in a quartz sample holder. The light scattered from the sample then passes through two lenses to reach the detector. I prepared rRaman samples in the form of frozen sample pellets (Figure X9), using a syringe to inject the sample one drop at a time into liquid nitrogen, which has a temperature of -320 °F. If you notice a resemblance between this sample and a particular frozen treat, you're not wrong—this is similar to how Dippin' Dots are made! Making samples in the form of pellets means that they can be poured directly into the sample holder, rather than needing to freeze the sample in a glass tube. Glass warps the baseline of rRaman spectra, making it difficult to distinguish signal from noise, so removing the glass significantly improves spectra.
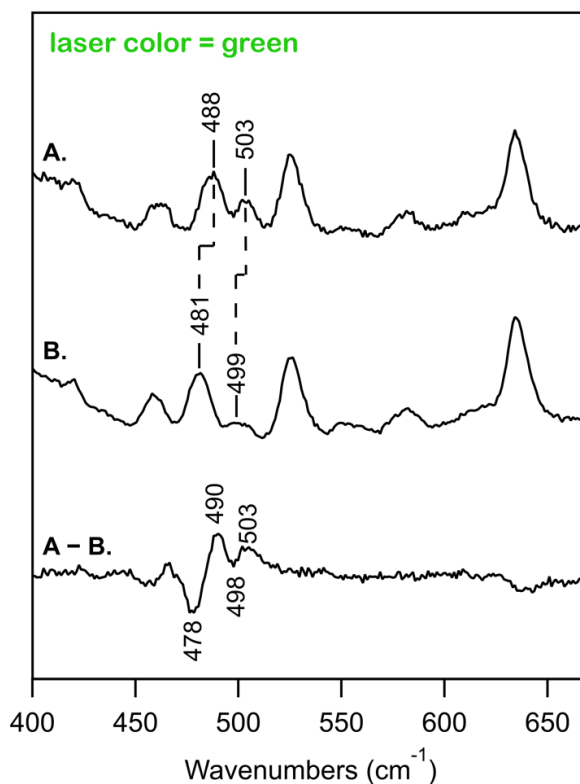
Figure 7.10. rRaman spectra of vitamin B_{12} using a green laser, where (A) corresponds to normal vitamin B_{12}, (B) corresponds to a form of vitamin B_{12} containing a heavier isotope of carbon bound to Co, and (C) is the difference between the two.

I used this approach to collect rRaman spectra for both regular vitamin B_{12} and vitamin B_{12} with a heavier isotope of carbon bound to the Co ion, and I was excited to find that there was a noticeable downshift of two rRaman peaks at 488 and 503 cm^{-1}. Finally, the elusive Co–C bond stretching mode of vitamin B_{12} could be identified! Using computational modeling and some math, I found that the peak at 488 cm^{-1} corresponds to a Co–C bond stretch and the peak at 503 cm^{-1} corresponds to a Co–C≡N bend (involving the nitrogen atom in vitamin B_{12}'s "R" group). This allowed me to calculate the strength of the Co–C bond, which turns out to be substantially stronger than the Co–C bond in other members of the B_{12} family.

7.6. Conclusions

The results obtained here represent a valuable step forward in our understanding of how to model and interpret spectra of vitamin B_{12}. For one thing, the new computational tool that I tested turned out to provide accurate and valuable predictions of experimental spectra, suggesting that it can be applied to future B_{12} studies. Being able to simulate the combination of electronic and vibrational excitations will facilitate more accurate interpretations of UV-Vis and rRaman spectra of B_{12}-containing systems. I was also able to identify the Co–C stretching peak in vitamin B_{12}'s rRaman spectrum, a piece that has been missing from the puzzle for a long time, which enabled me to calculate the strength of that bond. I feel very lucky to have uncovered such a fundamental aspect of vitamin B_{12}'s nature, and I'm proud to contribute to the foundation upon which future B_{12} researchers can build their work.

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