CI LABORATORY NOTEBOOK

VOL 31

Aug 26 2023

Working on Source of Coagulation paper.
Need magnification check.

$$\left(\frac{\text{measured cm (in meters)}}{\text{magnification}}\right) \cdot 1E6 = \text{size in microns}$$

or

$$\text{Magnification} = \frac{\text{measured object in meters}}{\text{size in microns}} \cdot 1E6$$

eg $[(1.4E{-2})(1E6)] / 7 \text{ microns} =$

$\frac{3584 \text{ pixels wide}}{12''} = 298$ pixels/inch vs 72 on older computer. Ratio $= 300/72 \approx 4.17$

So on the high res computer.

$$\text{Magnification} = \left(\frac{(1.4E{-2})(1E6)}{7}\right)(4) = 8000\times$$

exactly as anticipated.
So be careful what monitor you are using when you and how you determine magnification.
Newer monitor is 4 times more dense than the old computer.

Our list of accomplishments now includes:

1. CDB coagulation evident - microscopy - paper is written
2. Dissolved clot in NaOH - prepped for electrochemistry
3. USB photos of ultrasound impact upon clots
4. High level microscopy of US clot impact.
5. Not all clots are the same! Variable density & polymerization
6. NIR difference of three variable clots
   VAX, UNVAX, Deceased
   Density, chemistry, polymerization level varies
7. NIR Coffee filter VAX vs UNVAX blood difference
8. Ultrasound benefits are now evident
   Two stages 1. draw up proteins thru skin 2. Healy process
9. NIR analysis of ultrasound impact upon clots
10. Extended NIR study of embalmer clots
    Combinations and differences of VAX UNVAX deceased have been evaluated.
11. Cultury process is back in place and fully operational, productive and repeatable
12. Solid & Soluble forms of the proteins are now readily available.
13. NIR & VIS reagent verification of proteins are now BOTH available!
14. Organic chemistry course is active again w/ emphasis upon amine, amide & protein chemistry and polymerization.

15. Titrator is fully operational again. pH sensitivity of solid protein has been reestablished
16. Acid - base protein chemistry will be important.
17. NIR plots of both solid and soluble proteins are becoming available. Distinction of OH group in soluble protein a huge flag!
16. Initial characterization of solid form protein: acidic, iron-sulfur ~~protein~~ polymerized protein w/an expected high hydrocarbon content.
17. Distraction from the potato influence on cultures has been removed.
18. Filament network became visible in the solid protein isolated.
19. VIS spectroscopy is fully in place. Shift of valley in addition to peak shift can be of valley important.
Ratio analysis may be useful but shifts are more direct.
* 20. Crucial info.: VIS spectrometer gives the peak of the solid protein form @ 876nm. This is the aromatic amine and is crucial to distinguish from the soluble protein. This tells us that the 700-1100 overlap between VIS & NIR can be critical info!

Lab techniques now available or soon to be piloted

1. NIR
2. Electrochemistry
3. USB, mid & high level microscopy
4. Titrations
5. Culturing
6. MID YR Library
7. Centrifugation
8. Binocular microscope
9. Air quality meter
10. EMF meter
11. Surface VIS spectrometry RGB
12. High level VIS - UV spectrometry
    - Cobalt reagent development
13. pH analysis - ORP - Conductivity - Solution
14. Spectrum analyzer < 1 MHz
15. Oscilloscope
16. Oxygen meter?
17. Ion Counter
18. Ultrasound imagery!

Anticipated:

1. Refractometer
2. Portable VIS spectrometer
3. Improved culture w/ $FeSO_4$
4. Column Chromatography
5. Frequency generator - programmable!

What you do not have:

1. Mid IR
2. Gas Chromatography
3. Osmometer
4. Distillation
5. Vacuum
6. Burette & Sep funnel
7. Incubator
8. Refrigerator
9. Dremel
10. Periodic Chart
11. Chemicals
12. Fume Hood
13. Printers

Not bad in such a short time in such a small facility.

Now, what is the key plan developing?
(There are many steps along the way, each of which is likely to have its own importance.

1. Identify and declare that the CDB have been and remain the source of the primary problems in global health. This has now been done.

2. Culture the organism successfully. This is now also in place.

3. Isolate the metabolic products of the CDB culture, especially w/ emphasis upon protein.

4. Profile both proteins in any and all ways possible but with deliberation, haste and priority. Recall that which has already been done. You do not have infinite time for this. You have to identify the features of importance

5 Learn the chemistry of these profiles as rapidly as possible.

6. Learn how to affect the coagulation/clotting in ALL ways possible as soon as possible. Nutrient approach is preferred. NaOH known, US known, enzyme an immediate target.

Aug 26 2023 (cont)

We do now know a critical difference between the soluble and insoluble forms of the protein.

The solid form does have an aromatic amine that the soluble form does not. The soluble form has the OH groups as the alternative. A crucial distinction between the two protein form and the VIS spectrometer was capable of picky though, however weak, it detects it clearly and upon repeat!

The VIS plot is saved in the Aug 25 2023 folder along w/ protein verification.

For the soluble protein we can see the NaOH does precipitate out over time. Acid seems to clarify the solution somewhat but not completely, @ least both not immediately. Behavior actually seem similar to the solid protein.

Therefore, in both cases, solubility of the protein, soluble and solid is significantly affected by pH.

Both soled and soluble proteins form precipitates in alkaline solution. The soluble proteins comes are quite white, I think they both do. You need to keep collecting material to have enough to work with, as before.

Soled form has an aromatic amino in lower half of UV spectrum (upper VIS) and the soluble protein has an OH group.

Enzyme function @ an optimum pH.

Therefore if you start working w/ enzyme or the protein you will clearly need to keep track of pH also.

"Increased negative surface charge correlates strongly w/ increased protein solubility"
ncbi.nlm.nih.gov

A protein that has a acidic isoelectric point has a majority of acidic functional groups in the composition.

Amino acid can be polar, non polar, charged, etc

You need to understand pI and pKa

Protein Design paper from ncb.nlm.nih.gov is useful
"Protein Design from the Aspect of Water Solubility and Stability.

You can follow solubility threads deeply but characterizing the protein is probably what you need first.
Classification of proteins might be a worthy subject.
We believe we already have acidic polymerized protein w/ high hydrocarbon content. —

—

I did an incredible amount of work circa Aug - Oct 2017 on characterizing the soluble form of the protein.

We tried to gather info on
1. Molecular weight
2. Solubility, Polarity
3. Pyrolysis
4. Headspace, volatility (health risks here)
5. IR
6. UV
7. attempts on Amino Acid Identification
8. pH and pI
9. Precipitation Study
10. metallic nature
11. Index of refraction
12. Concentration - Dilution Model based on IOR
13. Conductivity vs Concentration
14. Vis reagent developed
15. ORP
16. Molecular mass idea

So you did a lot of work here to let you know how it can be uniquely identified & established uniqueness. However, what do you really know?

1. What is its general classification?
2. You know it is toxic but how much does that really help you.
3. You don't know anything about how it works, where & what you need to know.

You need to know the maximum no of ways that the protein can be degraded.

In terms of the "big plan" you can study chemistry and properties all you want, but essentially the only value of that will be if it helps you to degrade it. The idea is to be what you need.

Ok,

* 1. You need to purify it.
* 2. You need to be able react with it or degrade it.

It does seem to me that our first approach is with enzyme. You now know that pH is a part of the picture.

1. Concentrate, purify
2. Can you determine charge? as f(pH)?
3. Can you affect it w/ pH Enzymes? (pH)
4. Can you affect it any way beyond NaOH (toxic) and Ultrasound (not accessible to most)
5. From experience, Caution w/ any fumes. You know it is actually highly toxic

1. You should probably use milk as the control

2. Does the change in freq vary between 400 - 500 nm depend upon Concentration.

Milk Container mass: 4.98 gms 5.00 gms
5.51 gms total Δ = 0.51 gms
16.82 gms w/ H_2O added Δ = 11.31 ml
So our Concentration level is

$$\frac{0.51 \text{ gms milk}}{11.31 \text{ ml } H_2O} = \frac{1 \text{ gm milk}}{x} \qquad x = 22.18 \text{ ml}$$

So we have an equivalent concentration of 1 gm / 22.16 ml H_2O.

Dilution Ratio
30,000
40,000
60,000
120,000

But in a cuvette, we can just use a ratio of 100 ul in a full cuvette 3 ml

75

50

25

going into 3 ml cuvette H_2O

and see how it behaves

Milk Trial:

Here is the data for the shift - concentration problem

Conc.	λ	Abs	Δλ	Δ Abs
Reagent	436.4	.3871		
100 ul	450.8	.9310	14.4	.544
75 ul	439.4	.6222	3.00	.235
50 ul	437.9	.5650	1.50	.178
25 ul	434.8	.4391	-1.60	.052

The shown gap of concentration is low enough, the shift can be observed directly, but it is still a measurable shift. It also gives a sense of the minimum detection threshold

Shift as well as Δ absorbance are directly a function of concentration

Use Lists 8 & 9 & 10

8 = Concentration

9 = Δ λ

10 = Δ Abs

Parabolic is sufficient over these ranges

8 & 9 $r^2 = .955$ $ax^2 + bx + c$

8 & 10 $r^2 = .961$ $ax^2 + bx + c$

Ok, so we learn that shift in λ and Δ Abs are both quadratically related to the concentration.

eg 4 times the concentration leads to 16x the absorbance approximately.

Therefore having sufficient supply is important. We should be fine. Lets determine Concentration as a 1/4x ratio

We have stock solution of 1gm / 22.18 gms H_2O = 1/22.18E-3

For diluted sample we have 1/ $= \frac{x}{1E3}$ x = 1.50

100 ul 22.18(30K)E-3 = 665,400

75 ul " (40K)E-3 = 887,200 x = 1.13

50 ul " (60K)E-3 = 1,330,800 x = 0.75

25 ul " (120K)E-3 = 2,661,600 x = 0.4

This says that we have a detection system on the order of 1-2 ~~PPM~~ Parts per Thousand not bad @ all for Vis spectrometry & would say. Eg on the order of .1 ml of protein is a liter of water.

Ok, this helps to know what we have. Absorbance is a log function so it will max out @ a certain concentration level, actually fairly low.

Aug 27 2022

Collected NIR on residual leg & leg lesion scabs.

(3) Strong
(1) Weak

909 Methyl (908) (3)

1186 Methyl (1194) (3)

1456 NH Primary Aromatic Amine (1452.5) (3)
OH Water (Not likely) 1453
NH Primary Aromatic Amines (1459.5)

1511 NH Amide/Protein (1510) (3)
Polyamide (1515)

+1700 (Out of Range) Methyl groups likely (1700 – 1736)

The assessment here is that there is likely an encrusted form of the polyamide that has erupted from the skin. It has a history of 6+ months longevity. Extremely painful and very difficult to remove even in fluid stage. Likely to be a concentrated state of the polyamide (protein) solid form.

Aug 27 2023 (cont)

You can do a whole lot of things, and they are all curious and important but in the end what you need to know the most is how can you degrade these proteins? You know that they are responsible for blood, skin and so many problems but people now see the importance of blood coagulation and blood clotting. You know that they all come from the same source + that is these fungi proteins

So the priority has to be to attempt to degrade these proteins. Enzymes are a useful lead idea. You now know that pH will go hand in hand w/ enzyme but it may be difficult to ~~last~~ change pH of the body but there is certainly an idea. But what pH to measure? Urine seems like your only real practical indicator. How are you going to calibrate pH?

Your work on the protein concentration curve yesterday is going to be important

You have no idea what the actual concentration of protein is, so it will have to be relative. You can certainly compare it to milk as one reference however. The soluble protein is going to be easier to work with since you have so much more of it. Let's start to purify it. And determine pI.

—

Very cool improvements. You now have a titrator that is perfectly set up as well as a calibrated pH meter. Our water quality meter was just a perfect complement. The probe has a smaller footprint and it can read pH calibration solutions. Finally now fits perfectly in the 50 ml beaker you needed.

Ok, we need to establish some reference for the soluble protein. Assume we work w/ the soluble protein first to begin with. We need to have a baseline of status @ some point. It will be a different starting concentration call time and we only need to know relative impact.

Reference parameter could include
1. pH (probably the simplest)
2. TDS
3. Electrical Conductivity (that meter is calibrated)
4. Mass
This could give us some sense of what we are starting with.

Beaker mass is: 34.85 gms
8 pipettes, Mass is now 59.53 Δ = 24.68 gms.
pH = 5.57 The TDS meter also gives
EC = 16.85 mS temperature.
TDS = 7625 PPM
This does give us a baseline of the state of this soluble protein. It will vary each time.
Now we introduce varying concentrations of this into a cuvette and determine absorbance curve and shifts as of yesterday. TDS may be an simplest way to estimate baseline concentration.
We want worry about precipitation yet
Lets go with 200 ul (1), 150 (2), 100 (3), 50 ul in the cuvettes & add 300 ul (4)

VIS spectrometry w/ reagent is going to be very helpful - I suspect I could create other reagents for other reaction types.

Ok, our curve look just perfect. VIS spectroscopy is going to end up being very valuable w/ protein analysis.

Sample/Concentration	nm Valley λ	Min Abs	from Control Δλ	Δ Abs
Reagent Control	430.9	.4011		
300 ul	446.7	.7915	15.8	.3964
200 ul	445.7	.6808	14.8	.2797
150 ul	440.1	.5541	9.2	.1530
100 ul	437.8	.4894	6.9	.0883

Abs + λ are equally important if not more so as it verifies protein existence. Absorbance only reflects relative concentration.

Δλ is logistic, which is actually an exponential, or logarithmic.

You can certainly use logarithmic over the range. It will be apparent.

$\Delta\lambda = -33.84 + 8.83 \ln(vl)$ $r^2 = 0.90$
Logistic is better however, and more realistic

* $\Delta\lambda = \frac{16.71}{1 + 10.89e^{(-.0194\,vl)}}$ MSE = 1.65 nm
There is a very decent fit.

* $\Delta Abs = \frac{0.4273}{1 + 34.014\,e^{(-.0204\,vl)}}$ MSE = .00015
There is a superb fit.

Ok, now we have good reference shift and absorbance charts.
Now your next move is to take an arbitrary amount of the soluble protein, add enzymes (or whatever you are going to add) and then take 300 nl, maybe 200, 150, etc and see if the curve changes (ie, decreases) after incubation.

Let's keep track of what we add.
Container = 12.61 gms
2 Pipettes = 9.00 gms Δ = 6.39 gms
(1 pipette = 3 ml).

Now we add enzymes of 0.025 gms.
Total mass 9.039 gms
Incubate @ Room Temp.
Stir w/ toothpick. Time = 1430 Apr 27 2023

You have a very good system develop here. It does not take very much material to conduct a trial. Any water soluble trial can be conducted w/ the arrangement.

—

Next we try the other enzyme.

2nd Trial

Container 2.53 gms

w/ protein 9.49 gms Δ = 6.96 gms protein

w/ enzym 9.54 gms Δ = .05 gms enzyme

Stir w/ toothpick & incubate

Trial started @ 1500 Aug 27 2023

—

There is a problem w/ using VIS to test for effects of enzyme upon the protein.

Why?

Because Enzymes are Proteins! How would you even separate the effect out?

You will need to use Microscopy & NIR for each enzyme & try and detect a difference.

Our numbers are however.

Sample/Concentration	Valley λnm	Min Abs	from Control Δλ	ΔAbs
Reagent Control	431.6	.3803		
300 ul Protein + Enzymes	450.6	0.7394		

—

I see no difference whatsoever w/ microscopy between w/ enzyme (small) and without. Water soluble protein is packed w/ CDB but no dark pol protein segments visible. Recall that it has been filtered also.

Even NIR requires caution because there too you need to separate out influence of enzyme alone. by itself.

—

A very important deduction and discovery tonight. We have identified tyrosine as likely one of the, if not the fundamental amino acid within the soluble protein.

The process:
We know that we frequently are using an "aromatic amine" and polypeptide in the NIR work. Polypeptide means protein.

So one of the questions is, how many aromatic amino acids are there. There are only three.

Phenylanine, Tryptophan, and Tyrosine.
Now, they all have an OH group which would increase solubility, but only Tyrosine has the OH group attached to the aromatic ring.

So give that 1 point in favor.
Next, look @ the role of each in the body.
Tyrosine is org a - L DOPA, dopamine, norepinephrine & Epinephrine. Precursor to melanin. PRECURSOR to the thyroid hormone thyroxine.

Give it another point in favor.

But here is the Clincher.

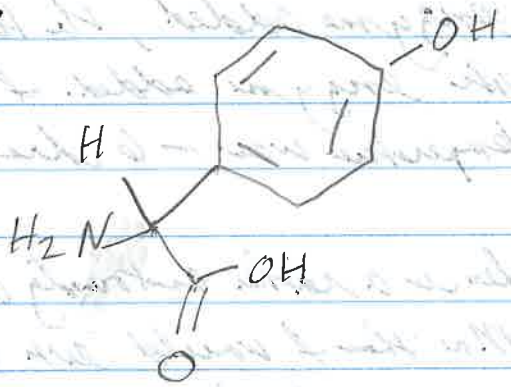
Tyrosine absorbs UV @ 274 nm
Tryptophan absorbs @ 280 nm

I measure the soluble protein tonight. 300 ul in the 3 ml cuvette.
I have good clean absorbance spectrum with a defined peak at?

You guessed it: 274.1 nm on the money
Certainly no peak @ 280.

We have tyrosine as a fundamental constituent of the soluble protein.
This is the type of information that I am often.

Notice it actually has two OH groups.



So much the better

This is a major accomplishment.
Only because of the culture.

Aug 28 2023

OK, potentially another very important finding. We know now that the soluble protein is dominated by an aromatic amine, and we have identified that as tyrosine as it has been generated by the CDB Culture.

I have made an enzyme impact trial ("small enzyme" tablet). VIS spectroscopy could not distinguish anything because enzymes are themselves proteins. Microscopy could not detect anything visually.

However, the NIR DIFFERENCE PLOT, ie reference is soluble protein without any enzyme added, the target sample is with enzyme added. Incubation @ room temperature was ~6 hrs.

There is a rather astounding difference and finding. More than I would ever have expected. Without question, the dominant influence is the removal of absorbance @ ~1472 nm

And what is 1472 nm?

An aromatic amine group! We have

1470 Primary amides R-C=O-NH_2 (1470) (3)
1470 OH Methanol (no history here)
1472 N-H Primary Aromatic Amine
1471 Amide / Protein

This is saying that the enzyme had a huge impact and degradation of the primary protein dominant in the soluble protein. This looks to be successful beyond your wildest dream. Only NIR detected this, & have no other means known to identify this change @ this level. This is potentially very significant.

Three prospect now exist for degradation
1. NaOH (toxic) dissolving clot
2. Ultrasound (physical degradation)
slow, painful, & difficult to access
3. Enzymes — a highly desirable strategy.

Now we notice that the dried sample has a very syrup texture and gloss. The question exists as to whether this is sugar based or not. The refractometer will help here.

Now, even though the treated and untreated w/ enzyme soluble protein samples both have high glow & syrup texture upon drying, nevertheless there is a major difference (lack) in absorption for the amide/amide.

This indicates that the glow/syrup texture is not resulting from the protein structure. Hydration content will need to be reviewed to avoid distorted investigation.

—

There is another signal of effect @ ~1546 nm

1546 NH. secondary amine (1545) (2)

and there is another @ ~1661 nm (2)

1661 Methyl (Halogenated Iodine) CH_3I (1661)
Methyl (Halogenated Cl) CH_3Cl (1661)
1664 Methyl ROH CH_3 (alcohol, Diol)

Now we don't know of a source for Chlorine or Iodine yet (but maybe electrochemistry will show them?) but we know that we have alcohol groups so methyl alcohol, diol candidates may have priority here.

All of these findings are potentially very important as they do show likely impact upon the soluble protein w/ the use of enzymes.

—

I now have a filament startup on the top of a culture. Three different organisms. I have looked @ them under the scope. They are the classic form. I have separated them off as a separate culture. I now have all previous forms in abundance and in the process of separation and purification.

1. COB
2. Water Soluble Protein - Tyrosine based
3. Solid Form Protein (paste?)
4. The Filamentous

The water soluble protein is the easiest to work with.

Let's take steps to purify this form and also work on determining pKa.

Beaker mass: 34.89 gms
50.64 gms w/ soluble protein condensed.
Δ = 15.75 gms

Control titration on titrator is very helpful.
You can flush the system w/ it.
NaOH - KOH
Will titrate w/ a H_2SO_4 solution w/ pH ≈ 10.
It required only 14 drops of NaOH - KOH
to produce 150 ml of H_2O @ pH 10.0.

*** Next we flush and prime the tubes and
set the drop rate @ a comfortable rate
and we take the initial burette reading.
Initial burette: 54.75
Now we titrate. We can titrate to pH 7.0
and to precipitate.

I will proceed w/ slow mode.
Initial pH of soluble protein condensed is 5.69
OK, we can ramp up speed to auto

OK, the titrator is getting very interesting and working
quite well. I have reached pH 6.76 and
need to switch to a larger container.
Burette reading @ pH 6.76: 9.0 ml
Δ = 54.75 − 9.0 = 45.75 ml used
so far. No precipitation yet.

Refill and reset burette ready.
Burette now reads 59.25 ml
Restart titration, re, continue in a large Container
I. have increased the drop rate since it obviously
is going to take a lot of base to neutralize the acid.
Remember our previous work identifying phosphoric acid.
pH 7.0 reached @ 43.6 ml Δ = 15.65 ml
+ Δ = 45.75 ml
Σ = 61.40 ml
used to reach neutrality.
total mass 15.75 gms
Ratio to reach neutrality is $\frac{61.40}{15.75} = 3.90$
Now it took 4 times as much.
Next time we use pH of 11.0 vs 10.0
Continuing. No precipitation visible
@ 2.25 ml pH = 7.36. No precipitation
61.40 ml + (43.6 - 2.25) = 102.75 ml so far
Refill to 47.50 ml.

We need to redo. Used too much capacity.
Let's use NaOH solution of pH 11 now.

Let's use 5 ml probe
Beaker mass 34.92 gms
w/soluble protein added: 40.00 gms Δ = 5.08 gms

Now let's make a pH 11.0 NaOH solution.
Let's use pH 10.5 instead. pH 11 is a whole lot (almost (10x) the pH 10.
Our volume is 1/3 also.

1. Prep titrant
2. Flush column and set drop rate into scrap container
3. Take initial burette reading.

Initial pH 5.85
! Initial Burette (59.5 ml) forgot to read it!

My goodness We may have an acid halophilic protein. Look like very high acidity.
pKa looks to be above 10.5 ?

Maybe not, they did precipitate out.
Maybe centrifuge alone is sufficient?

Ok,... thinking as usual...

It is not impossible that we have up to a half dozen different protein formation that are possible are involved here, when you think of the culture varieties that have emerged over time.

We have a "soluble" protein now and a "solid" form in our current generation of cultures, but these forms have very visible difference from the final cultures in Monticello VT. Let's recall all that you have seen. You also realize that you have a "potato culture" influence in Monticello that may actually end up being very important, and not just a "distraction". We will once again review our final culture note out of Monticello.

Monticello final version did exclude potato.

1. The solid form was quite lighter color, and had somewhat of a "curdled" appearance. It was never actually analyzed, all focus went on to the "soluble form.

2. The soluble form was a light green color (presumably Fe+2) and it gradually oxidized to turn a brown color.

However, the visual properties of that "soluble protein" are relatively different from what you are encountering here. Remember if dried sufficiently it would turn into a "tar-like" substance. Remember all the pyrolysis & gas chromatography work indicating high hydrocarbon content. Flame tests, etc. Toxicology studies, the neck health issue. This does not seem the same as our "soluble" form now. Remember when you heated that solution, a white precipitate form which you just discarded and did not analyze. That white precipitate might relate to what you are now finding in solution and calling the soluble protein.

3. Our "solid form" here has the consistency of a paste, (not ~~curded~~ curdled" and it is dark brown. We have not even begun to analyze that yet.

4. Our "soluble" form here deserves more separation attempts. There may be two forms w/in. One that stays suspended in solution until centrifuged, and then the remainder

5. The filaments are actually another protein expression form. We are just getting that form to emerge now. It is interesting that a yellow color is forming in the center of the emerging filament colonies.

So it is not impossible that we have, depending upon the culture medium used

1. A truly soluble form, greenish in tint, which is separated by mild heat heat to the solution
2. A "white precipitate" w/in that greenish tint solution that was ignored & may relate to a white precipitate that you are now seeing
3. Our current "soluble" form which may end up separating into 2 separate forms, one of those solid & one to truly liquid.

so possibly two here

4. Our current "paste", dark brown, which never actually has been seen before.
5. The filament structure as their own separate protein form, difficult to digest or breakdown as we know.

re-creation

There is a lot recreation and separation that needs to take place here.

One of first critical questions is what happens when you centrifuge our current "soluble" protein, let alone pKa precipitation analyses??

Are there two products, and if so, which one has the tyrosine?

You see now that
1. UV
2. VIS NIR overlap 700-1100
3. NIR 900-1700

are each going to be very important and must be used in a composite manner for all analyses.

* Also the idea of a "halophatic protein is not to be dismissed.

I am now centrifuging the so-called "soluble protein" of the current culture.

Are there two different products?

And the answer is yes, there are two products. One remains soluble, & there a small amount of material form on the bottom.

First pit stop: Microscopy!

The centrifuged material on the bottom is pure CDB isolate. It is a very good method of isolate the CDB or the need excess.

The remain solution will be the "Truly Soluble Protein" and the water will subject to precipitation and pKa analysis.

The CDB are so small that they remain in suspension but they are not dissolved.

Note "TSP" nomenclature

The lesson here is that the "Soluble Protein" that is separated from the culture will need to be centrifuged to be the "Truly Soluble Protein".

Both low value - it will be of interest to have NIR on both to see how they compare, It is easy to have crossed signals from lysis.

Aug 28 2023 (cont)

Here is something fascinating and unexpected. I have made a NIR plot comparison of the "Isolated CBB" from the "Soluble Protein" isolated via centrifugation with the CBB centrifuged removed, ie, the "Totally Solubed Protein.

I anticipated they might have some noticeable differences. They have none. They are essentially the same.

A bit difficult to explain this right now. In the section of the NIR spectrum it says that the nature of the CDB is identical to the protein that it is creating, at least the soluble form.

The UV tyrosine question would be worthy to see as well as VIS-NIR 700-1100 comparison would also be of interest.

But except for pKa determination and precipitation needs, it says the separation may not be changing that much. An interesting situation

The general plan:

1. Keep isolating and purifying protein type and learn what you can about their chemical nature and how they are different from one another.

2. Try any mitigation idea you would like on any protein acquired. So far, NaOH, ultrasound, & enzymes

3. Establish the chemical linkage between the CBB, the advent of coagulation and clotting, any skin or ailments, and the coagulation/clotting products. ie, show that it all stems from the CDB.

See if your enzyme sample no longer shows tyrosine in the ~~UV~~ ~~VIS IR~~ UV section @274 nm, 280 etc?

Aug 29 2023

And actually it's pI not pka...

—

I have two major objectives today:

1. Does the influence of enzyme on the TSP (Totally soluble protein) change the absorbance of tyrosine @ 274 nm?

2. Can we precipitate the TSP via titration?
   Can we scale the experiment small scale just to save materials.

3. Begin to take advantage of how
   UV 225–400 nm
   VIS-NR 700–1100
   to try and learn more

—

A current effort. I am running UV on the TSP (Totally Soluble Protein)
My first run (used 300 ul) gave an erroneous and noisy plot. Was very noisy.

I repeated the run, this time w/ 100 ul
& have a perfect run. Not sure why the difference, i.e., what happened at the

first run.
Now what I do have are two definite UV peaks:

276.3 Tyrosine (weakly acidic)
Fairly broad peak from 274-278.
Definitely centered near 274 & not 280.

Tyrosine also stated to be at 275, not 274
Tryptophan 280
Tyrosine 275
Phenylalanine 258

231 also a strong peak, sharper in profile.
"UV absorbance @ 230nm is known to be sensitive to protein conformation".
Nucleic acid are traditionally quantified @ 230, 260 & 280nm

"UV spectra of protein solutions usually show slopes, not peaks, @ 230nm".

But guess what, we have a definite peak.
"The peak @ 230nm is due to absorbance by peptide bonds"

* "A_{230} is a valid & convenient probe to determine thermodynamic stability and unfolding kinetics of proteins w/ many potential applications"

This gives us heightened interest in any alteration of A_{230} as well.

"The peaks @ 280 (approximate - CEC's) result from absorbance by rings of aromatic amino acids (tryptophan, tyrosine, & phenylanine."

The UV comparison of TSP against enzyme treated "soluble protein" is uncertain @ this point. There does seem to be some shift at two wavelengths:

The control ~275 nm peak shifts left to 273.5
A valley of 255 on the control shifts right to 257 nm.
The peak @ ~231.5 nm remains constant for both control and enzyme treated.

The NIR plot shows a major difference in the polyamide absorbance. The case of UV is thus far less well defined. It will repeat using TSP for both and also maintain similar concentrations.

Now lets compare between 700 - 1100 nm. There is no significant difference between the VIS - NIR overlap region.

Now let's prepare enzyme into the TSP solution.
Only TSP - Totally soluble protein will be
used for testing in the future.

Well I have certainly cut to the chase.

The [redacted] Enzyme (large enzyme) completely
alters the UV spectrum of the Totally Soluble
Protein. There is fully amazing. Time of
enzyme incubation @ room temperature is 72 hrs.
There is every thing you could hope for.
So far we have evidence of

*** 1. Small enzyme significant NIR alteration
*** 2. Lg enzyme major UV alteration
* 3. Ultrasound degradation of clots + skin proteins
Ø 4. NaOH does solubilize (eventually) of polymerized
Clot - the is not viable - lethal

There is what you needed to know.

Tyrosine a major player in the TSP.
Never could have been done w/out the lab.

Let's see if we can scale the titration problem.

Beaker mass = 20.59 gms
Mass w/ TSP = 23.62 Δ = 3.03 gms

Drops NaOH pH
11 OK, then easily precipitated [redacted],
however it required centrifugation to
determine it had taken place.
We can only estimate 20(.06 ml) = 1.20 ml
so maybe 50% of the volume should be sufficient

Since it requires centrifugation to detect, what
is our best titration design

You could start w 5 ml pattern.
Add 10 ml H_2O Σ = 15 ml
Estimate 2+ drops

Beaker = 20.75 gms
+ ~ $\frac{5}{10}$ ml ⇒ 30.78 gms Δ = 10.03 gms

56.75 Burette Start
Start pH 5.05 Try to use a flashlight

@6.15 pH, 6.5 6.75 yes starting to precipitate.
Magnifying glass + flashlight.
Yes, this is it 6.75 ± 0.25 pH

This idea & method worked quite well.
We want a NIR Card of the soluble protein TSP
against the large enzyme

Aug 29 2023 (cont)

We now have the difference plot of the large enzyme (Large Enzyme) upon the TSP. Same phenomena as w/ small absorbance.

A major drop in absorbance @ 1473 nm

Difference regions (can be greater or less than reference)

Reference = TSP Target = TSP w/ Large Enzyme 72HRS

1 = weak 3 = Strong

1134 (1) CH Aromatic (1142)(1143)

Nothing else around

1472 (3) Amide (1471)

Aromatic Amine (1472)

1548 (2) Secondary amine (1545) R-NH-R

NH from secondary amide in proteins (1550)

1661 (2) Halogenated Methyl (Cl or I) 1661

Clear evidence again of amine/amide disruption

Aug 30 2023

I have made great improvements in the lab today.

I have set up a completely operational chromatography column today. I call it LPLC vs HPLC "Low pressure liquid chromatography". Set up stand, clamps, counterweight, aquarium pump for moderate pressure (absolutely essential over gravity feed), fine white sand & gravel caps. It is all marvelous - all my historical development took place w/in a couple of hours. It looks like a perfect column set up.

I also now have portable visible light spectrometry again to replace the failed PASCO. Thanks from Vernier - I do like their products. This is going to help out on the road quite a lot.

I also acquired Ferrous sulfate!, rubber stoppers, & great beakers & flasks, syringes as burettes

Also today I have precipitated a relatively large quantity of the soluble po protein. It looks quite pure and this avoids the need for continued centrifugation. This definitely looks like the way to purify this product.

Increasingly, however, I am curious about the solution that remains after solution. It has a yellowish color to it and yet is very transparent. This looks to be another product in its own right and need to be examined as another possible protein form. You literally may have a Otis half dozen varieties of proteins.

1. $FeSO_4$ culture had clear green after mild heating
2. $FeSO_4$ had curdled white form - potato?
3. We have dark brown paste
4. We have white precipitated protein
5. We have clear yellow fluid after precip
6. We have filaments

Pure $FeSO_4$ Culture and potato influence will be approached soon.

Control study on "sugar water", NIR, and glossy surface will be investigated soon.

Clear transparent solution will be investigated soon.

White precipitate will be separated and pH tested for solubility.

The brown paste & the curdled forms and the filaments are an entirely different matter.

Mid IR library may be used @ some point to clarify.

I also have acquired some great study courses and books as well:

1. Organic Chemistry Textbook - Pearson
2. Essential of Biochemistry - from India - 2 Version
3. Structure and Mechanism in Protein Science
4. Video - OSU Biochemistry Course - Kevin - Great Courses
5. Courses & Duke University Courses
6. Great Courses - Nature of Matter - Chemistry
7. Udemy Organic Chemistry
8. Pearson Organic Chemistry & Regular Chemistry

The precipitated protein very slowly settles out of solution. A very good separation takes place here.

pH of separation is ended ~ 6.5. Initial solution is pH ~ 5.7.

I can already tell that the clear yellow remaining solution of the precipitation is its own product. The "sugar water" does not account for the glossy texture of the majority of NIR samples than for.

From the current culture medium (sugar, fertilizer, & mineral salt) we already have four distinct forms showing up, each of them almost certain to be protein.

1. The dark brown paste
2. The precipitated protein in suspension @ pH 6.75
3. The clear transparent yellow solution remainder
4. The filament structure

This is now to be compared w/ the FeSO4 culture whereas I only made two separations and may have missed the precip as its own entity of importance.

—

I have two quick methods of protein determination now. UV & VIS w/ reagent.

Here is our UV result for the clear yellow solution after precipitation:

1. We have a defined peak @ 272.6 nm
This means that we have tyrosine / protein (?) in the yellow solution. This now actually becomes the "Truly Soluble Protein" TSP (?) Maybe Not!
as the precipitated protein has been separated from it. Both are significant components of the CDB solution culture.

Now we do not have the peak @ 230 nm as we did before so this indicates that indeed a separation has taken place.

Now lets go to VIS reagent method.
But before this, capture 700-1100 VIS-NIR overlap region.

The yellow separated solution, ie TSP is where the gloss comes from, NOT the sugar water.

The TSP is likely our polymer material as studied w/ MID IR extensively in the past.

There is NO VIS IR overlap absorbance from 700 - 1100 nm, at least of significance w/ the instrument. NIR will be of much interest.

Now on to VIS reagent tests for protein.

Well this is getting interesting. There is almost no shift w/ the reagent (~1 nm) and also the absorbance magnitude is almost identical to the reagent

Then, w/out NIR information, is indicating that we have tyrosine in solution but not at the point any confirmation of a protein. Visually we have the indication of a polymer film surface.

So @ this point our best information is that of a water soluble polymer that contains tyrosine, but not a protein. Need NIR work.

Definitely getting interesting. Tyrosine is stated to be of low solubility in water. Well just maybe this is not water.

"Water soluble polymers are substances that dissolve, disperse or swell in water, and thus modify the physical properties of aqueous systems in the form of gelatin, thickening, emulsification / stabilization".

Examples are: polyethylene glycol, polyacrylamides, polyacrylic acid copolymer and polyvinyl alcohol.

The water soluble polymers carry out a variety of functions in aqueous media including stabilizers, thickeners, flocculants, coagulants, film former, binders and lubricants. Sound familiar? I have thought of it as a water based protein; it may be a soluble polymer instead.

Aug 30 2023 (cont)

You have made another very interesting discovery based upon a bit of nap brainstorming. It centers upon the pH upon which the protein precipitate out of solution ~6.75 vs the original pH of ~5.7.

The discovery is that if you take urine pH it might well behave the same way, and it does. My urine sample pH was 6.15. I added NaOH to bring the pH up to 6.75 and then eventually to 7.0.

There is indeed a precipitation reaction that takes place and the urine turns cloudy. The precipitate is white as is the CDB culture solution.

If you centrifuge the sample you will see

A white precipitate @ the bottom.

* Methinks it is the same, but time will tell.

* This may therefore be an additional mitigation strategy – try to raise urine pH to 6.75.

Aug 31, 2023

We have something very important to figure out. Upon precipitation by pH of the "soluble protein" there is a very clear separation between the precipitate and the transparent yellow solution that remains. In addition, after adequate settling, the precipitate accounts for more than 5% of the full volume.

As the vast majority of the solution is that of the transparent yellow solution but very small but almost certain minor fraction [illegible] likely extremely important.

Our job, however, is to characterize each of these forms that embody the whole. The first take is that the precipitate is protein based and the yellow solution, thought to also be primarily protein, was most recently assessed to likely be a polymer.

* We now need to find out what really is the case here.

Yellow Solution Only for Now:

Our methods of analyses are going to include:

1. UV
2. VIS w/ reagent } spectroscopy — These are measurements
3. NIR
4. VIS-NIR overlap

4. Drying of solution to low level water content.

5. Microscopy
6. Chromatography if helpful
7. TOS, Conductivity
8. Polarimetry if actually available
9. Water for fumes, pyrolysis, etc
10. Effect upon live blood?
11. Enzyme activity?

Let's start

(Addendum: The "paste" protein did start a yellowish colony on it after setting for about 1 week).

Evaporate the yellowish solution in the water glass to concentrate it.

The solution is much more transparent after setting for 24 hrs so that will likely affect the spectrums. Consider anhydrous

thus far as highly tentative.

This tentative NIR spectrum gives us peaks at

1203 - 1204 (2) 1200 OH from water (maybe)
1195 methyl
1211 methylene

1470 NH from primary amides R-C=O-NH_2

all open 1471 - 1473 (3) 1471 N-H Amide/Protein
1472 NH primary aromatic amine

1540 Starch, Polymeric alcohol

~1545 (2) 1545 NH Secondary amine R-NH-R
1550 OH of water and an overtone of NH of the amide groups of ovalbumin

Now visually this NIR plot appears to be the same as that of the solution prior to precipitation and more thorough settling, but we know by physical form and appearance that they ARE DIFFERENT, so we must identify that difference.

Enzymes make amino acids.
Polar & non-polar separation of amino acids is really helpful.
(you have positive & negative polar sub groups)

We have a really interesting UV spectrum from 226 - 400 nm. Our reference is water. We actually have a negative absorbance from ~226 - 209 nm! This finding has been repeated.

This seems like a very curious affair to me.

In addition, we have two very distinct peaks:

290nm (2) relatively weak but sharp & definite

302nm (3) very strong peak

Some factors that might affect negative absorbance

1. The blank contains "something that absorbs in that wavelength region
2. Unmatched cuvettes
3. Fingerprint on the blank cuvette

NEGATIVE ABSORBANCE INQUIRY

OK, we did find @ least the main problem. It was either:

1. The water used as the reference OR
2. The set of cuvettes used had a problem or were not matched. BOTH

We will figure out why.

In the meantime, what we have is actually high absorbance @ 263 – 285 nm and that is broad.

Sub Peaks are @ 264nm & 285nm

UV absorbance of proteins range w/ peak absorbance from 260 - 280 nm. There is rather a close match w/ what we have. The ways we do have proteins. Notice 274 of tyrosine is not exactly on here, however.

—

Now we read an article that says the 280 absorbance actually only work for proteins that contain tryptophan or tyrosine residues. They are important. They page also says that protein not containing tryptophan or tyrosine can be measured using 205 nm. 205 is lower than I can measure, I can only measure down to 226. [Tyrptophan = 280, Tyrosine = 274] Wikipedia

So what you are concluding here is that if you have a definite peak @ 274 you can say that you have tyrosine. But otherwise absorbance in the 260-280 region is characteristic of proteins in general.

So conclusion here is that by UV we do have a protein, but that we do not associate them it to tyrosine.

Now we also see that our cuvette or water had a problem, so lets see why or which.

OK, the problem was w/ the cuvettes!

A big lesson here. When you work w/ UV the cuvettes must be known to be matched and that they are indeed suitable for UV.

In the future, we will keep very close track on all cuvettes and carefully reserve them by used for UV.

* I think we can see that you simply can not tell what you have by the style of cuvette, you must know exactly the source of the cuvette when you are working w/UV.

Keep UV cuvettes separate and marked as such.!

Ok, now we have a suitable UV spectrum of the yellow solution and it says we do have a protein. Visual behavior still says a polymer so best assumption is now that of a polymerized protein. This corresponds to our earlier historical work.

We continue to evaporate the yellow solution. This is going well and must be monitored every hour.

Now lets go for the color protein reagent test. 400 - 500nm

Reagent Control = 434.0nm
w/yellow solution added = 438.0nm < 3min
438.5nm < 6min

As the color shift is almost immediate. The magnitude of absorbance is almost the same so magnitude shift is not a reliable indicator of presence of protein, only concentration.

Our conclusion: We have a detectable shift in λ. Therefore conclusions w/both UV and VIS reagent continue to support the existence of a unique protein form.

Evidence therefore continues to accumulate for the existence of a polymerized protein in the yellow solution. Let's measure pH.

I shall follow up w/ NIR.
Everything is saying you have 4 different protein types now. That will be a sig nificant finding if it continues to be confirmed

This is probably the main goal for now.

I think that we should always purify the "soluble protein" now since we know that it is composed of two proteins, one highly pH dependent.

* The TSP does produce Volatile Organic Compounds (VOC) detectable @ ~.05 (.05-.08) mg/m³

It does have an odor.

This protein is the most likely form used in the toxicology studies - Pyrolysis
Biological Neck Harm
VOC's in human health are mostly blood borne

and have chemical signature that are known to correlate w/ specific diseases.

The pH is the same as after titration, or 7.0. This makes sense. No reason for it to change.

The TDS measurement is 7750. Very high. This is the same as prior to precipitation.

Next: Electrical Conductivity = 17 mS + also reasonably high - on the order of that of blood.
So it is a conductive polymer it would seem w/ high levels of dissolved materials.
In Organic electrochemical analysis might tell you quite a bit about what these are.

Probably the next logical stage of presentation is to announce the isolation of the four variation of proteins, without going in to great detail on each.

Some general defining characteristics of each might be appropriate.

Properties:

1. Solubility
2. Charge? PI pKa is the method here, determines it
3. Visual? - Microscopy - Morphology
4. Acid-Base Chemistry - pH sensitivity
5. Isoelectric point
6. Melting point

Hydrolysis is the breaking down of a molecule by reacting w/ water.

HCl acid is the most common method HCl 6M

PI is the pH @ which a protein has zero net charge. When the pH is higher than the isoelectric point, the protein has negative net charge and when lower, positive net charge.

I think if we photograph each protein the USB used and acquire an NIR of each protein that it will suffice for presentation purposes.

Sep 02 2023

I now have the programmable signal generator in place and operational along with picoscope oscilloscope to monitor the output up to 1.5 MHz. Output can go up to 60 MHz. I also have test leads set up to introduce into a medium such as a culture. This is a very intriguing prospect. Recall that you made an ELF detector @ one point and this would be possible again. But not in the cards right now. Culture work is.

Capabilities now:

1. NIR
2. VIS
3. UV
4. NIR-VIS overlap

Spectrometry

6. Chromatography
7. Programmable Freq. Generation & Oscilloscope (to a microscope slide?)
8. Ultrasound - Imaging & Signal Generation
9. High Quality Microscope
10. Electrochemistry
11. Portable VIS spectrometry
12. Culturing
13. Titration - pH
14. Centrifugation
15. Air Quality
16. Microwave
17. Mid IR Library
18. Education Courses
19. Binocular Microscope
20. USB Microscope
21. Refractometer Coming
22. EMF Monitoring
23. Conductivity & pH (Crude Titration)
24. CO_2 Monitoring

Portable Capability:

1. NIR
2. Non VIS spectrometry (Protein detection)
3. Moderate microscopy
4. Electrochemistry

Quite possible:
1. Ultrasound imaging
2. Centrifugation
3. Signal generation & oscilloscope
4. Refractometer - Index of Refraction
5. Air Quality
6. EMF Monitoring
7. Conductivity & pH (Crude Titration)
8. Education Courses
9. VIS NIR overlap

Chromatography is one of the most powerful low cost technology but having a column & stand will be difficult to achieve.

Now that you have a fair amount of Capability

(What you are missing:

1. Mid IR
2. Gas Chromatography
3. Osmometer (Refractometer alternative)
4. Polarometer
5. Distillation
6. Fume hood

Not bad, considering all is in UT!

the question is what is so next. I think that you systematically need to characterize each protein. What characteristics would you like to use and are they universal for each protein.

And why do this? So that we have a sense of what we are working with.

Candidate Characterization

1. NIR. We actually already have this and you have a great deal of first level information w/ this analysis.

2. UV little information but helpful
3. VIS reagent - protein detection
4. Solubility
5. Acid Base Chemistry
6. Inorganic Analysis - Electrochemistry
7. TDS
8. P_i determination?
9. Conductivity
10. pH in native form w.r.t. Concentration
11. Response to DC or AC signal?

There is a very good list.

Let's start the process.
Probably best to select a method and then go through all proteins.

1. NIR has been collected on each protein.
Very useful info.

2. Go to UV and work in some solubility along the way.

Sidenote: Urine development over 24-48 hrs is to be examined under scope.

On the urine, I did not expect it to be so flagrant. Bold, blue filaments are visible in numbers w/ a single drop of urine. Incubation period was 72 hours (or less may have been sufficient).

Let's go to UV on TSP.
TDS and electrochemistry and native pH would be good general measurements.

TSP: TDS is 9270 PPM
pH = 6.2 (after precipitation!)
~20mS - meter exceeded.

NOW DILUTED diluted by a factor of ~~4~~ 3 15ml 5ml
EC = 17.75 mS H_2O TSP
pH = 6.39 (after precipitation)
TDS = 6952
Definite prominent absorbance @ 274nm = Tyrosine

Parameter List - TSP:

Now in place

0. Index of Refraction - Refractometer
1. NIR
2. UV
3. VIS reagent
4. Solubility
5. pH
6. TDS
7. Conductivity
8. Acid Base Chemistry Reaction
9. Inorganic Analysis
10. PI determination
11. Response to DC/AC Signal

Reagent shift: Reference 433 nm
Shift to 436 nm

Protein existence confirmed.
Greater Concentration (3x) causes a shift to 442 nm, as expected.
Used 300 ul in cuvette.

Baumé: 21.6 ⇒ Specific Gravity ≈ 1.175
Quite High

This refractometer by MilWaukee is a bit tricky to use. It may be that light affects it a lot more than the other.
Consider covering it when you calibrate

I think that it is much more sensitive to light reflections. The light must stay on for a full second + for it to actually work. Short bursts of light tell you that it is not working.

Urine Baume' = 2.2 much much lower than the protein

Water soluble & quite clear.

TSP: Acid Base Chemistry:
No strong visible reaction to subjecting the TSP to a strong acid or a strong base. The strong base (NaOH, KOH) does cause the solution to become a bit cloudy - not expected from purification impurities from the pH precipitation process.

Now let's start looking @ constructing a titration curve.
So it seem that we would record volume vs pH. Also we need to know the concentration of what we are working with.

You also need to start @ a certain pH to begin with to know that you encompass the range.

First we'd would suggest we acidify the TSP to a ~~low~~ low a pH as is practical, the begin to observe behavior w/ pH.

—

Further we see is that it actually is quite the challenge to get the pH of the titrant up to pH 11. The Caustic KOH-NaOH commercial solution Carbon to max out @ ~ pH 10.5. The, however, is quite strong as you can see. It will be adequate for our use now.
Our titrant pH = 10.7 ≅ 11 as we last prepared prior w/ NaOH.

I will now take 10 ml of TSP and dilute it by a factor of 2, ie 50% TSP, 50% H_2O, to total volume 20 ml.

Now let's drop the pH as low as is convenient w/ H_2SO_4.
Initial pH of analyte is 6.86
Mass of beaker + analyte = 55.80 gms
(Large scale reads 56 gms - good)

Strongly acidifying the protein does lighten its color.
I am able to get the pH down to 3.51 w/ strong H_2SO_4. Now let's titrate.

Record volume regularly w/ pH.
Mass of beaker is now 56.85 gms (w/ analyte).
We have therefore added 1.05 gms of strong H_2SO_4.
Concentration may be available from MSDS (Often 10M)
Original Titrant Volume 57.75 mL

Volume	pH	Vol	pH	Vol	pH
57.75	3.48	26	~~4.35~~		
56.5	3.50	23.5	4.36		
52.5	3.55	21.0	4.36		
49.25	3.60	19.5	4.36		
44.0	3.70	17.75	4.37		
36.0	3.90 ??	17.75	6.79 ??	Switched Containers	
30.0	4.2	Point definitely passed!			
27.0	4.28	See notes next page.			

Well it certainly looks like our pH is going to be on the order of 4.36 - I have recollection of that point in past work - we shall see.

The layout of the titration seem to be great. However, the pH changed dramatically when we shifted to a larger container. I do not think contamination should be a significant factor. We did somehow miss the titration point however.

We will repeat this in the large containers to begin w/. Volume + Concentration seem to be well balanced.

Also notice that once the solution became acidified it has never returned to its darker color, even upon going back toward a neutral pH.

Repeat the trial. Same conditions + measurements
Initial pH = ~~3.36~~ 3.34
Initial Burette = 57.5 mL

The trend we paused for last reading, ~ 1 min

TSP Titration

$\Delta = +49.75$

	Vol	pH	Vol	pH	Vol	pH	Vol	pH
			RESET VOL					
"3"	57.5	3.32	"3" = 55.25	= 3.87	3.5 = 57.25	9.04	57.0	10.38
1 min	54.5	3.31	✓ 51.0	3.93	54.0 enlarge container CLOUDY SOLUTION	9.28	49.75	10.40
"	52.5	3.32	✓ 46.0	4.02	50.5	9.62	41.75	10.43
	49.0	3.34	✓ 41.5	4.10	44.5	9.91	29.75	10.46
	44.5	3.39	✓ 36.0	4.22	39.5	10.04	15.0	10.49
	36.5	3.46	✓ 30.25	4.43	35.5	10.11	3.75	10.51
	27.75	3.57	✓ 25.0	5.04	30.75	10.18		
	21.75	3.64	✓ 22.5	6.31	21.75	10.27		
	15.25	3.73	✓ 17.25	8.0	14.5	10.32		
	11.50	3.79	✓ 13.75 (264.0)	8.30	8.5	10.35		
"30"	5.5	3.87	9.75	8.67	4.25	10.38		
$\Delta =$	52.0 ml		"30" 3.5 (=253.75)	9.04	4.25 = 57.0			

30 5.5 = 355.25 $\Delta = +49.75$

3.5 = 303.5 − 49.75 = 253.75

Proteins are generally the least soluble @ the pI (Remember the "cloudy" acid base test w/ NaOH result?) At a pH below the pI, proteins carry a net positive charge. Above their pI proteins carry a net negative charge.

In practice, a protein w/ an excess of basic amino acids (arginine, lysine & histidine) will have an IEP > 7 (basic).

A protein w/ an excess of acidic amino acids (aspartic acid and/or glutamic acid) will "often" have an IEP < 7 (acidic).

2D electrophoresis can be used to separate proteins (but you have already physically separated them...) via the culturing process. Quite good work...

Therefore solubility is our primary method to determine the ρ IEP.

So it seems to me that TDS, conductivity & specific gravity would all be useful to measure the solubility level.

But how does IEP determination relate to the titration curve of protein?
Milk-Casein would also be a good test case.

Let's work on a way to determine turbidity/opacity of a solution. VIS - UV spectrometry?

—

Adenote - Recall that urine looks sufficient to produce an advanced filament culture. This would be worthwhile reporting on.

Also notice that precipitation occurs with urine in an alkaline state. This is saying that we are able to produce the precipitated protein in urine fairly easily and this would therefore bypass the culture process. This gives us two out of four proteins w/out culturing.

1. Filaments
2. Precipitate Protein
3. As the TSP there also in low concentration
4. ~~The settled~~ - Tyrosine - UV is the answer to that
4. The settled protein form we don't know enough about to detect or identify or establish the existence of.

—

Back to turbidity - in UV we have tyrosine @ 274 nm. The lower the absorbance level is the more insoluble the protein is. Therefore when the curve flattens out we have likely found the IEP of the TSP.

Sep 6 2023

There are ~~3~~ 5-6 projects going on:

1. Titration of the TSP. I have a good method in place. 274 nm
2. An effort to determine the pI of the TSP (UV absorbance vs. solubility)
3. Purification of additional TSP under culture
4. Collection of additional "settled" protein
5. Examination of solid material settling out of TSP over time. Microscopically.
6. Incubation of filament protein.

—

I notice after titration and alkalenization of the TSP to pH ~10.5 that UV absorbance at 274 nm is not existent, as hoped for and anticipated as a measure of lack of solubility of the TSP. However, I also notice that significant absorption does exist @ 230 nm.

"UV absorbance @ 230 nm is also known to be sensitive to protein conformation"

"Absorption in the 230-300 nm range is dominated by the aromatic side chains tryptophan, tyrosine, & phenylalanine"

"Graphene oxide is often quantified via its UV absorption, typically @ around 230 nm". "The authors report that the absorbance of GO is size dependent." Study is between 1-2.2 um."

Question: is GO soluble?
"Graphene oxide is a water soluble carbon material in general. Oxidation of graphite turns black graphite into water dispersible layers of functionalized graphene oxide related materials".

This is significant. You now have candidate

- @ 230 nm of
- 1. Folding proteins
- 2. Aromatic side chain protein but not just tyrosine
- 3. Graphene oxide

We are working from the protein perspective but we will now also keep an eye on the graphene oxide property.

- Here is what is happening w/ the TSP after incubation of ~ 1 week @ room temperature. It is forming crystalline structures that settle out of solution

Under the microscope, these crystalline solids do not look exactly natural. They are [illegible] characterized by sharp right angle structures and cracks and even an acute angle. It looks too variable to be a natural process @ this point & will try to incubate a sample under warmth and observe.

—

Now, let's do the titration again, but this time we are going to be looking for the point of maximum insolubility. We are going to try to use pH or absorbance as our measure to see if it reaches a maximum. I will extract 3ml cuvette @ regular ΔpH interval and evaluate. The cuvette sample will be returned to the container.

Now, to do the Ca, we do not need to acidify the TSP further as we see that the solution is transparent and the

Initial pH was 6.46. We saw the change of dramatically the tyrosine absorbance by acidifying the solution to begin work. So we start w/ the native TSP solution pH.

Let's measure UV 226–300 nm to start a reference.

I see some unexpected results. The UV profile of the TSP looks different. We now have peaks 292 & ~265 w/ considerably lower absorbance @ 230 nm.
I must wonder if the crystallization process observed underway now is changing the nature of the protein.

For now, we just monitor the UV profile for change & lack thereof.

Scan	pH	A_{266}	A_{274}	Scan	pH	A_{266}	A_{274}
Abs 1	6.44	1.979	1.979	9	8.30	1.809	1.806
2	7.04	1.863	1.862				
3	7.10	1.846	1.850				
4	7.36	1.838	1.839				
5	7.69	1.835	1.830				
6	7.96	1.828	1.826				
7	8.10	1.820	1.816				
8	8.20	1.808	1.809				

We have it. The last 2 curves are the same.

	Vol	pH	Vol	pH	Vol	pH	Vol	pH
	56.25	6.44						
slow	56.0	6.66						
	55.9	6.85						
	55.7	7.04						
	55.3	7.10						
	55.0	7.36						
	54.9	7.69						
stabilizing	54.0	7.97						
	53.25	8.10						
	52.75	8.20						
	52.0	8.30						

absorbance is indeed decreasing 226-300 nm w/ titration increasing pH.

Cloudiness of TSP is already in place @ pH I.P. 7.10

Let's choose absorbance @ 266 nm (widest spread) as our solubility indicator.

Keep in mind that our analyte is getting more dilute as we add titrant so absorbance might decrease also from that.

—

We actually accomplished exactly what we planned to do. Our last two absorbance curves coincide @ pH of 8.20 & 8.30.

The means assuring the pH meter remains calibrated OK, that we have a pI determined for the TSP @ 8.2.

The implies that the protein TSP has a excess of basic amino acids (arginine, lysine or histidine). At pH below the pI, the protein carries a net positive charge. (Remember previous work w/ histidine focus?)

OK, what are the implications of this?

Things w/ TSP we may already know

1. Tyrosine present
2. Basic amino acids present, assume histidine
3. Polymer nature
4. Water soluble
5. @ Biological pH, ie ~7, the protein carries a net positive charge - implications re: the ?
6. Crystalline geometric rods develop @ pH ~6.8 under incubation for ~ 1 week. (Also polymerized)
7. Highly conductive (indicates high ion content)
8. We have NIR plot to analyze
9. We know to be less soluble @ pH > 8.2
10. pI estimated @ 8.2
11. Likely subject to enzymatic disruption
12. Likely subject to ultrasound disruption
13. Emergence of graphene oxide vs protein use @ 230 nm AFTER titration alkalenization

Sep 07 2023

There is a slew of projects that can be active so as usual I need to prioritize:

1. I have purified & collected 3 of the 4 proteins:
   1) TSP
   2) Precipitated
   3) Settled.
   4. I am lower in amount of the filament protein than desired.

2. I have improved the incubator (the plate version)
3. The "crystal" precipitate emerging from the TSP is more than curious. It is hard to conclude any other than it represents synthetic biology. The images are astounding and perplexing.
4. I have prepared a NIR card of the TSP "Crystal" (a very big understatement). Drying ...

Many many ideas of tests to try:

??
1. Does the TSP crystal form react to current or frequency?
2. How should I create more filament protein sample?
3. The graphene oxide is intriguing, how would you have a test for it?
4. Urea NaOH precipitate make TSP precipitated protein?

Separately for the moment such questions as "synthetic biology" evidence and graphene oxide questions, you have established a priority and desire to chemically characterize each potential type.

Of your list for TSP, what remains?

1. NIR analysis of TSP
2. Inorganic analysis of TSP
3. Response of TSP to EM as freq, DC, AC?

NIR data is on RV PC.

Inorganic analysis would tell us what minerals are in solution. This would help address the "crystal" issue.

Sep 08 2023

I am now incubating the second generation TSP to see if the exotic crystal development can be accelerated. It took approx 1 week @ room temperature or cooler.

—

I now have NIR plots, side by side, of all your prototypes:

1. TSP
2. Settled Protein
3. Precipitated Protein
4. Filament Form

And now I have added the TSP exotic crystal form/polymer upon drying to the set. It is very useful in that the primary activity change seems to be a shift in the range of 1465-1490 nm. There are other changes that will also be tracked to.

1465-1490 change represents changes in amine, amide, polyamide and aromatic amines.

What this means is that we are seeing variations in the protein forms, and the makes sense.

Time is a factor here, ie incubation

from 1480

The TSP exotic crystal has shifted to ~~1465~~, 1472
This now appears to most likely center on
the creation of a ~~secondary amine~~, aromatic
amine from a possible polyamide.
You now understand the difference between the
varying primary secondary & tertiary amines
so this will have more meaning in the future.

~~We also see a shift from ~1540 to ~1535~~ VOID
~~This might involve changes between polymeric~~
~~alcohols, polyamides, secondary amines,~~
~~or amide / protein. The secondary amine~~
~~is consistent w/ the 1465 shift noted above.~~

You also notice shifts in valleys. You
has seen this is important w/ protein detection.
You would like to learn to interpret and give
more meaning to a "shift in the lack of absorbance"
vs the existence of absorbance. They
both are going to have value and you has ignored
this factor thus far.

Now recall our priority now is to focus on characteristics of the TSP – not the TSP exotic crystal. So one thing @ a time and all in due course. But the previous does give a preview as to what to expect and what to be looking for. But start thinking in terms of lack of absorbance also.

So on the TSP we have.

916 Methyl (CH_3) 915 (2)

~1001 Aromatic Amine (1003) (2) (Remember Tyrosine!) 174 nm
Alcohols (1004, 1006, 996) Phenol 1000

~1206 OH from Water (3) less likely possible (1206)
Methyl (1195) Methylene (1211)

1481–1482 Polyamide (1480) (3) (but polyamide is protein also)
Secondary Amine (1481)
* Amide/Protein (1483) 2nd overtone N–H, $CONH_2$

We know that we have protein

Note some 2nd overtones do occur in ~1000 nm region!

1574 1570 NH Amide Amide/Protein (1570) (2)
1570 Poly amide

OK, this is good. Verification of protein, tyrosine
Visible Polymer nature. Water soluble. pI known.

Two steps remain
1. Inorganic atom analysis
2. EM signal into protein DC? AC freq?

Let's go w/ electrochemistry - ACV
Ok, I am running again w/ ACV.
I have recovered previous similar / desirable data files. Conditions of run are:

t equil Ø
E begin -3.5
E end Ø
E Step .01
E ac Ø.2
Freq 50Hz
Scan Rate .03

n=352 datapoints
1 mA has marker.
time of scan 2 min

Ok, our graphs are not very active here, but it is active in one very important way.

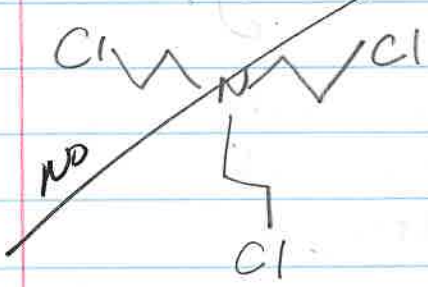
y^0 ACV y^0 DC y' DC y' ACV

-3.32 (-12mA)!
+3.33 (22mA)!
-Ø.68 (+3.5 to Ø) (20uA)
(No reversal)

$\frac{3}{2} N_2(g) + H^+ + e^- \rightleftharpoons HN_3$

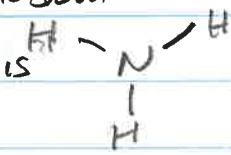
ie, Nitrogen activity - presence is strong.

This reaction seems to be very reversible.



?? Nitrogen mustard

This is confusing. NH_3 is

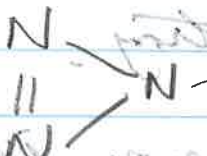


Sidenote: The crystal development w/ the TSP is going to be an important topic. TSP02 is generating CDB-crystal complexes w/in ~6 hrs of incubation @ ~85°F. The crystals thus far are more conventional in appearance @ this stage than those of TSP01, but they are also more heavily and obviously infused w/ CDB internal crystal activity.

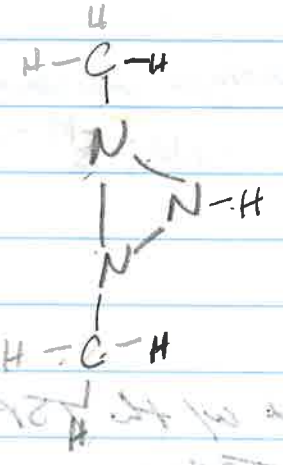
A ~~the~~ reaction is $3N_2 + H_2 \rightarrow 2HN_3$?,?,? In this case it is hydrazoic acid but this is w/ the H_2, not H^+

This seems to be a lot of confusion about HN_3.

Another formula

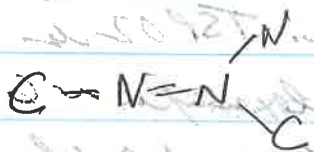


Somebody called this formula triazirene and also someone states that it is highly unstable and that is also wrong.

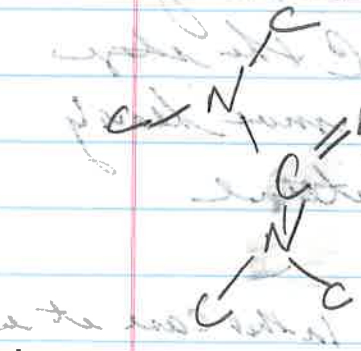


$H_3C N_3 H CH_3$ Is this possible?
Seems like a secondary amine
Does this compound exist?

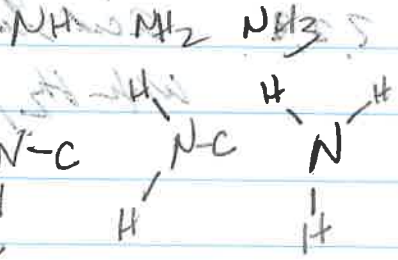
$C_2 H_7 N_3$



Dimethyltriazene
does satisfy - Not the H.



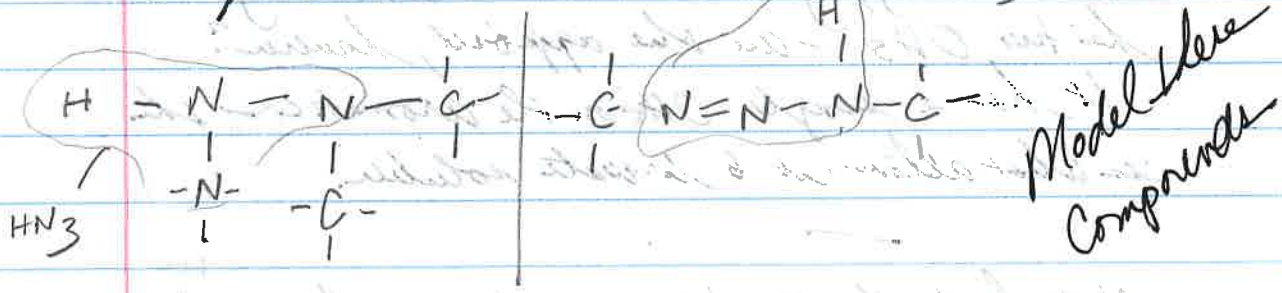
Methyl guanidine



NH NH_2 NH_3 ; Amines

What is shocking to catch my attention here is the reversibility of the reaction. The loss of an electron, or oxidation says that N_2 & H^+ are in solution. That makes sense, N_2 gas should be present and we know the solution is acidic which is H^+. But going the other way says that HN_3 ~~HN_3~~ (or whatever form) exists in solution as well.

Also lets look @ the amount of current involved. And among the reactants from the other direction INITIALLY should demonstrate whether the HN_3 already exists. That is important to know ...



Model these Compounds

Work up these Compounds w/ more than hydrocarbons

~~-0.66~~ -0.68 We want to look @ also.

And why would not iron show up since we know we have the hydrated complex?

-0.68 Candidate. If our value matches, it
Now we have a minus is oxidation if reverse sign,
then it is reduction.

Candidates: H_2O_2

Oxygen: ~~Hydrolysis of water~~ +.695

Sulfur SO_3^{2-} -.66

Platinum Chloride - remote

Selenium remote

Carbon - Benzene Ring +.70

Nickel Remote

Rhodium remote

Now, we do know that we have an aromatic rig, Tyrosine; We also know Tyrosine has two OHs - are the opposed, however? It has a single OH on the aromatic - this is what allows it to be water soluble.

Next look for oxidation - reduction directions
We have a -0.68. Reaction is listed as positive. Therefore the listed reaction is reversed. It therefore represents the decomposition of H_2O_2.

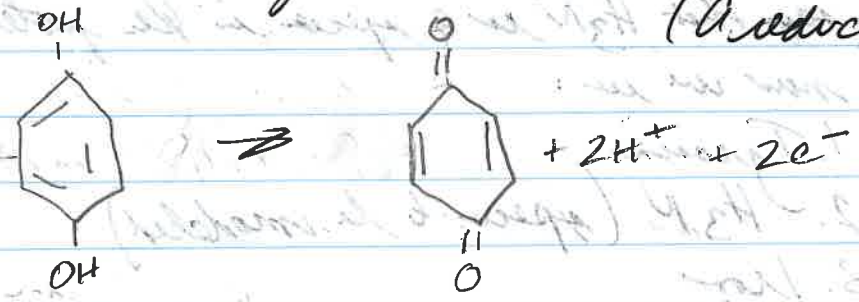
$H_2O_2 \rightarrow O_{2(g)} + 2H^+ + 2e^-$ (a reduction) ~~of O_2~~

Not reversible @ this point.

This says the H_2O_2 would already be present in the TSP.

In the Carbon-Aromatic situation, the value is listed as +.70. This is positive. Our moment is negative. Therefore the reaction is reversed.

(A reduction)



This would therefore proceed to a less stable compound that is no longer aromatic. What type of compound is this?

Sulfur:

The value shown is negative, −0.66

There it matches and is an oxidation reaction

$$SO_3^{2-} + 3H_2O + 4e^- \rightarrow S_{(s)} + 8\,OH^-$$

This means you start out w/ a sulfite (I believe, will be checked) and you end up w/ a solid form of sulfur.

Each or all of these exist as candidates.

We could have all three of them

Notice that the DC curve is as insightful, or more so, than the AC curve in this ACV protocol being used. Both are helpful.

There is no shortage or decrease of the reaction @ +3.33 V w/ a run from +3.5V to Ø.
This says that H_3N is a species in the protein.
So now we see:

1. Tyrosine
2. H_3N (species to be modeled)
3. Iron
4. Acidic (Phosphoric acid after peroxide research)
5. Basic amino acid w/ PI (Histidine? by previous research)
6. Polymer
7. Exotic Crystal - Potential Circuitry Development via CDB complex.
8. Highly conductive
9. 3 Candidate species @ ~~Ø.6~~ -Ø.68V

not mutually exclusive:
1. H_2O hydrolysis
2. Aromatic Reduction
3. Sulfide to Sulfur oxidation

10. Highly toxic
11. 2nd protein species within (precipitated out by pH)

?? 12. Electromagnetic susceptible?

Sep 09 2023

A few preparatory items done this morning:

1. The inoculated TSP 02 Crystal cultures have been examined under the scope. At least four forms are observed.
   1. CDB, stand alone
   2. Dominant feature in the crystal formation, w/ a definite CDB internal complex developing.

The title and subject is now revised to:
"The Towers of Exotic Crystal Biology: The CDB".

I have never heard of a term "Crystal biology" before, but I have at least

Guess what? There is a likely related subject by the title.
"Macromolecular Crystallography" - the study of artificially grown biocrystals.

"Biocrystals" "Protein crystallisation"
Variables: Concentration, temperature, pH
X-ray diffraction
ESRF European Synchrotron Radiation Facility
- generate X rays
There is one very hot related topic - high level.

Ok, you have now tapped into a recognized leading topic of biological research:
"Biological Crystals"
indeed w/ an emphasis upon protein characterization. Primary tool of analysis is X-ray diffraction to produce a visual signature of a protein.

2. Item 2: I have transferred the filament sample into a fresh active culture medium that is ~40 hrs old, as this is how the filament structure first appeared. I am trying to enhance growth.

3. You need to settle the cover slip issue. Take 2 cover slips, soak one in strong acid & the other in strong base. Rinse & try a live blood inspection via a fresh commercial slide.

Setup is:
1. 1 Cover slip in strong acid solution – ~~5~~ 10 min
2. base
3. water

After 5 min, rinse all cover slips in water 5 min. Let dry & wipe clear before use. Test live blood.

* I have done several things to clarify matter & Catch up today.

1. The cover slip question, at least for my work, has been settled. There is no contamination on my cover slips that affects results in blood.

2. "Biological Crystals" topic is now quite viable & a very hot topic.

3. Ebay refractometer issue is being dealt with.

4. Telomerase culture has been transferred to a fresh medium in an effort to accelerate growth.

5. Last night you made a read in the NIR plot of the crystal form.
NIR is going to get more interesting because different plots are going to be heavily used and the valleys will be as important as the peaks.

* I would suggest our current course of action on TSP is to:

1. Form a difference plot from TSP & Crystal TSP.

2. Set up a fast run on freq generation/electrolysis into the TSP.

Drying.

Sep 10 2023

There are now three papers to be written on the TSP:

1. General characteristics + model proposals
2. Crystal Biology
3. Mitigation proposals - laboratory based.

—

With NIR, I see that I can leverage the info more further by combing last direct and different plots to the maximum advantage possible. With three samples involved, the generator 6 NIR plots to work with. Quite a lot of info. Then you can also consider MIR work in conjunction to construct models.

Diff plots:	Ref	Target		Samples:
	TSP01	TSP02		TSP01
	TSP01	TSP01 Crystal	(leave in place)	→ TSP02
(leave in place)	TSP01 Crystal	TSP02		→ TSP01 Crystal

Now stepping through each scan (6 scan sets)

1. TSP01 Direct
2. TSP02 Direct
3. TSP01 Crystal
4. Δ TSP01 - TSP02
5. Δ TSP01 - TSP01 Crystal
6. Δ TSP01 Crystal - TSP02

Now, then 6 way plot, even though they are all essentially the same compound, might and seem to be able to reveal differences between the samples as desired.

The is done now by selecting any two samples out of the three @ a time. You show only those two direct NIR plots & then the difference plot simultaneously. 3 out of 6 plots are then you shown @ one time.

Our first case is TSP01 vs TSP02. We know from the lab work that TSP02 was exposed to higher temperature during culturing, higher than desired. & anticipate the sample was degraded to some extent in the process as it was darker in the color and showed some signs of mold growing.

Now here is what the plots say:
What we do is look for PEAKS AND VALLEYS on the difference plot.

Now the next statement is we look only @ the difference plot and enlarge it to essentially become normalized. Peaks and valleys readily show up the way. (1) Weak (3) Strong

(+) ~914 Peak (2) Methyl (915) Increase
(−) ~979 Valley (1) OH from water (979) Decrease

(+) ~1129 Peak (1) ArCH (1142) Possible: Broad Peak Increase

(−) ~1200 Valley (1) OH from water (1200) Decrease

(−) 1462 Valley (3) Secondary Amine (R-NH-R) Decrease
VERY STRONG DECREASE HERE

(+) ~1534 Peak (1) Secondary Amine (R-NH-R) (1530) Increase
Amide / Protein (1530) Ammonia in water (1534)
(+) ~1660 Peak (1) Halogenated Methyl Polyamide (1538) Increase
(~1660)

We are often several and prominent info primary. 1462 Peak is quite strong. This is exactly the type of info I am after. Recall that the difference plot is not a mathematical constructed difference - IT IS A MEASURED DIFFERENCE PLOT and therefore likely to be quite real. It has the potential of highlighting the existence or absence of functional groups that were NOT DETECTABLE in any way on the original direct NIR plots. Vly promising method to gain additional structural information.

These results are surprisingly consistent and may indeed be telling. They suggest strongly that:

1. TSP02 has less water in it than TSP01 (plausible since sample was excessively heated)

2. TSP02 has a much lower protein content than TSP01 (also reasonable from excess heat)

3. Methyl groups and Aromatic hydrocarbons may have been affected, as amino, protein, and ammonia production.
Some chemical differences as experts to exist, also not surprising w/ excess heat, and it is thought anticipated that TSP02 is a somewhat degraded version of TSP01.

Not bad for being totally unsuitable except for darkened color clues.

Now, as next plot of interest is TSP01 vs TSP01 Crystal
The web will display the 3 curve plots.
TSP01, TSP01 Crystal, and the Δ plot.

Now hide the direct plots. Show the Δ plot only.

924 peak (1) Methyl (915) Methylene (930)

1136 peak (1) ArCH (1143)

1320 peak (2) No match

1466 valley (3) R-NH-R Secondary Amine (1465) Decrease
Amide Protein (1483) alcohols (1464, 1468)
Strong decrease here

~1548 peak (1) Secondary Amine R-NH-R (1545)
Secondary Amine (1550)

1656 peak (1) Methyl Nitro (1654) Halogenated Methyl (I or Cl)
1661.

1678 valley (1) Methyl (1678) Ar-CH (1680).

These indicate decrease of protein ratio - directed into crystal?
Aromatic CH & Secondary amines possible.

So we do see some differences here also that may be
helpful to interpret the crystal forms as they
develop and are studied further.

There is exactly the type of experiment in that I am after. Even if there is uncertainty about species groups, and less uncertainty about others, we know at least that there are differences of some sort, & also have other means (eg TOS, pH, Conductivity, refraction etc) to establish differences if need be.

There is an advantage similar to UV on Chromatography: You may not be able to see the difference but at least you know that there is one and you can continue from there.

There are already 3 papers in store on the TSP:

Repeat Electrochemistry

1. ~~General~~ Characteristics of the TSP - Model Development
2. Crystal Biology
3. Mitigation Prospects

Enzyme study is required for mitigation prospects.

Next, we see that the latest culture looks to be very successful. We clearly seem to have an acidic fermentation process in place; the odor seems to match vinegar. Apple cider vinegar is indeed a fermentation process.

Remember our match on mid IR w/ Kombutcha(sp)?

Also see filament colonies in the latest culture, it's certainly our unlikely hero.
We will let the culture evolve to a longer period.

—

What we do right now is monitor the crystal morphology status of TSP01 & TSP02. TSP01 now has only abundant settled crystal layer. TSP02 has a thin opaque layer.

—

We also need to test if the culture is producing alcohol.

* When a hydroxyl groups is directly attached to an aromatic ring, the resulting group is a phenol, not an "alcohol".

* Tyrosine has a phenolic OH group as a side chain

"Tyrosine is a versatile amino acid that plays an important role in regulating the structural conformation transitions of proteins; its redox-active property also facilitates interactions between metal ions in the active centers of enzymes and the transport of electrons along w/ protons".

—

OK, Crystal monitoring.

Start w/ TSP02 spare layer now settling in the culture. Compare it to the incubated sample.

TSP02 72 HRS.: Crystals are not as sophisticated or as well developed as in TSP01. However, the rectangular Crystals are now also clearly formed. COB-Crystal Complex seems apparent. New "beaded Chain" microorganisms appear; size estimate ~ 1.25 micron vs. CB COBC ~ 0.4 um. Room Temperature Culture.

TSP02 Incubated ~ 96 hrs. There seems to be no real advantage & the cultures appear to be dominated by COB presence. No crystal formation seen. Will not pursue @ this time.

—

TSP01 → Now incubate @ room temp for ~ 9 days. The crystal formations can become large enough that so they can no longer be retrieved by suction into a pipette. Possible w/ suction on the top of the pipette, however. What is happening is that the crystals are congregating into dense mats. They don't look very healthy by the way; a different set of spikes and daggers. Clearly a significant change is in progress.

—

I think you need to hold off on electromagnetics for now.

Working w/ Enzymes probably more important right now.

Also repeat the electrodemetry. With the raw broth. Metal signatures?

to the issue is that you probably need to provide reports before you get too far along. You will get lost in the details.

The most important step now is to see if you can repeat the enzyme experiment. You do now have two separate samples to work with.

Enzyme treats are started @ 1645

Now fortunately I have saved my previous enzyme sample so I am able to repeat that run as well. This is very fortunate.

Now, just so we realize it, with the original enzyme solutions set by ~ 1 week, the "small enzyme" solution of TSP 01 shows filament colonies (2) emerging from the top of solution. NOT a good sign.

Scan 1 In addition, the UV run of the same solution still shows a peak @ 274 nm. Also not a good sign.

"Large"
Enzyme solution (original). TSP01 DOES NOT show a peak @ 274 nm. It has a monotonic decrease in absorption from 226 nm to 300 nm. The profile is different. There is a levelling off section from ~259 & 266 nm however @ 274 nm absorbance is on a decline.

Scan 2

The change now observed twice w/ the enzyme complex. Everything says that the "large enzyme" complex interferes w/ the metabolism of TSP01.

Very important news here. Now confirmed twice.

—

Next will look @ TSP01 after sitting for ~2 weeks. It still has the peak @ 274 nm.
Tyrosine Confirmed.
Enzyme impact confirmed.

Scan 3

—

Excellent work.

Next we look @ the "Large" enzyme impact after only 45 minutes exposure to TSP 0.

Wait on this → The peak is already shifting and is now at 272.3. The change is not yet clear & verified however. What is important is that a peak absorbance @ 229 nm is forming – this is a strong sign of protein folding now taking place.

Under the semi period, ≤ 1 hr even of tyrosine holds the same, we have definite evidence of protein folding taking place.

* → a definite change

* 228.7 shifted already & 229.4.

No sign of ~274 shift as actually in place.

Repeat Comparison from scratch.

As another Control, put just the enzymes in water to see if they produce a peak @ 230nm.

—

2nd run is a little more ambiguous after 1½ hrs.

Both enzyme forms show a shift from a small peak @ 227 (TSP01 original) to a more pronounced peak @ 228 nm.

—

OK, here is Control enzyme run info:

Large enzyme is showing a peak in H_2O @ 266.4 and @ 232 nm. This is all going to end up being important. After more settling, the 266.4 peak disappears. The peak @ 232 nm remains. Definitely shifted from the large enzyme treated TSP 01. I still see an effect there @ 228.5 vs the enzyme Control @ 232 nm

The large enzyme TSP 01 solution with a week digestion has a different profile from TSP 04. The peak @ 274 simply does not exist. This says that sustained exposure to the large enzyme disrupts the protein structure and specifically the tyrosine component. When you think about it, there no longer is any digest peak absorption from 274 to 280 so the implication that no protein w/ the aromatic functional groups still exist. You might have a protein but not with the aromatic side chains. In addition the peak @ 230 does not show the charge, and thus further indicates disruption to the protein structure. Remember that the "small enzyme allowed for filament growth AND also returned the peak @ 274nm. Everything is saying the large enzyme has disrupted the protein.

Now let's follow up w/ what a reagent test says about protein existence.

Above will allow more time for the large enzyme to react.

Sep 10 2023 (Cont)

I have no doubt now that I will be able to establish whether the TSP has been interrupted or not in its metabolism. I have more than enough methods in place now to detect change.

1. UV spectroscopy
2. VIS spectroscopy
3. NIR
4. Microscopy - esp Crystal detection or lack of it

And from there we can go on Electrochemistry, TDS, Conductivity, Specific gravity, etc

The is the main goal now. Can we demonstrate interference in the TSP metabolism (beyond ultrasound)

Continuing: With the reagent test using the control enzyme solution (large enzyme) we have a very clear shift both in magnitude and wavelength. We see a

Clarence says from 432 to 440 nm. The interpretation of this, coupled w/ the UV scan so that we still have protein in excellence, but that it no longer contain the aromatic side chains such as tyrosine.

This is now further evidence that the original TSP protein has been interrupted in a fashion.

Now lets go back to the new enzyme solution now standing for 5+ hours and see how they look before. Then we will inspect under the scope for crystal biology, where has now become an identifying feature of the TSP.

Scan 1 is TSP01 + Large Enzyme

Our peak is at 272.5

~~Our peak is @ 272.5 nm. Definitely a shift to the left~~ The instrument has a deviation of 0.1 nm. We also have a definite strong well defined peak @ 230.1

400 ul put into the cuvette now rather than 300 ul.

Ok, the 272.5 peak conclusion is a NO GO at this point.

Our control TSP01 has its peak @ 272.2 There is not a significant difference.

What is different however is the behavior ~ 230. The control TSP01 has a weak peak @ 228 nm. The large enzyme 5½ has been a stronger and pronounced peak @ 230 nm.

This indicates the prospect of protein fold changes.
We must keep going. Let us now try a microscopy on the enzyme solution. We must collect from the bottom of the vial as the crystals settle there.

We know we still have protein – the question has it changed and can we show that it has changed?

More tests on the enzyme is also required.

Ok, a critical test has been passed. Microscopic examination of the TSP01 Large Enzyme 1 week + solution shows NO CRYSTAL FORMATION. Plenty of COB but no CRYSTALS.

This is a crucial observation. It says that the protein sequence, the protein expression has been interfered with. This is the most conclusive evidence thus far. UV indication of faulty charge, prospective, is further supportive.

Now let's look @ the small enzyme 1 week + TSP01 solution under the scope.

Ok, the small enzyme 1 week solution also shows no crystals. However

1. The COB concentration is immense
2. Filament colonies appeared in the vial
3. The UV spectrum does not depart from the control spectrum as much as it does w/ the "Large" Enzyme.

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Qualitative tests in the CRC Handbook are going to be helpful. You already have a workable test for Fe^{+3} and Fe^{+2} w/ Ammonia.

Fe^{+3} w/ ammonia - red-brown gelatinous precipitate of iron(III) hydroxide

Fe^{+2} w/ ammonia = a precipitate of iron hydroxide.
Now iron(II) hydroxide is actually a White solid

but iron(II) hydroxide precipitate can have many many colors (yellow, brown, orange & red) mine is dark green (added H_2O_2 to H_2O)
O_2 gives a green tint.
Over time oxidation will turn it to rust or we know.

But emphasizes that pure iron(II) hydroxide is white in color.

Now what is interesting is our protein is yellow in color but originally is green until we alkanize it with NaOH & precipitate the protein. & therefore assume because of the yellow orange color that it is Fe^{+2} state.

However, adding NH_4 to the protein creates a dynamite precipitate, light in color, not red-brown gelatinous. Thus indicate the protein actually be more in the Fe^{+2} state.

Remember our purified protein before - it was always green in color as you never did alkalize it. (you alkalize a little as the very last)

What is useful here is that you have a reasonable useful test for Fe^{+2} & Fe^{+3} w/ only ammonium. Fe^{+3} red is more unique, Fe^{+2} seems to have quite a bit more variation. I would say our proteins is primarily a Fe^{+2} state. That actually does make sense.

Now let's test our enzyme solution again.
Combined w/ TSP 01 w/ UV, now w/ ~ 18 hrs elapsed.

—

Addendum: I have improved the efficiency of the cultivary process w/ less waste!.

1. The "settled" protein can be separated from the insoluble particulate material w/ a screen strainer.
2. Residual fluid from the settled protein collected can be used as a thin flat fluid culture to support filament growth separately.
3. The filament culture can be kept warm on top of the small incubator / fry.
4. The precip settled protein can be evaporated in the smaller warmer w/ the door partly ajar.
5. The TSP can be evaporated in large volume on the hotplate w/ gravel heated ~ 130°F. Don't let it get too hot this time.
6. Primary culture chamber ~ 85-95°F.

—

Now back to enzyme analysis.

Scan 1 = TSP01 (Week + w/ sygnificant crystal development)
Scan 2 = Week + large enzyme + TSP01
Scan 3 = 24hr Large enzyme + TSP01

Each of the scans have differences.
There is a significant question already as to the age and condition when the enzymes are added and if that makes a difference. It is now expected that it does make a difference as the TSP01 is fundamentally different than it was a week ago since the crystal stage is well developed. The test requires that you treat the enzyme solution when the TSP is fresh or new and crystals have not yet developed.
You need a fresh TSP batch, not overcooked, and you have one in the wings.

That being said, here is what characterizes each scan.

Scan 1 1. "Control" TSP 1 Week + w/ sygnificant crystal development.
1. Has a peak @ 273.5
2. Has a peak @ ~~229~~ 232

Scan 2 2. Week + TSP01 + large Enzyme
1. The peak @ ~ 274 is simply removed. Monitor as increase
2. Has a peak @ 229
3. 24Hr Enzyme + Week + TSP01 w/ Crystal

Scan 3 1. Peak remains @ 273
2. Peak @ 233.5

So what we do know is that the 24 hr enzyme exposure to week + TSP w/ full crystals is not behaving the same as the week + old enzyme solution. Therefore the enzyme compact has not been fully replicated otherwise the microscopy showed crystal inhibition by both large and small enzymes.

So the question is why?

1. TSP01 is simply not the same as it was a week ago. You need your goal better.
2. Higher level of enzyme required?
3. More time required w/ the enzyme 24 hr vs week + might make a difference.

There is a difference in all cases. Your done is to replicate #Can #2.

I have now started a test culture w/ starch added. The potato heals continue to peak my curiosity. Potato may seem to obstruct the issue considerably as a mobile second microbe appears, so I will reduce the question to one of starch.

I have now set cultures up as a two-tier process w/ the use of an oven rack screen. This will double the production rate - it will be quite helpful. I can set up any combination of either evaporating/concentrating or culturing.

You can use your bucket for some water in the time being.

What I want to recall here is my recollection of how dramatically and quickly the UV spike of the first run with the enzyme (large) on TSP01 was when the solution was new - pre-crystallization. This is what I want to duplicate.

I have added more TSP01 and increased the enzyme concentration somewhat. Not excessive & the have differences already

Scan 1 Control : Peak @ 272.6
No peak near 230.0

Scan 2 Large Enzyme + TSP Mixed Time, recent & 24 hrs:
Peak is at 270.9
Definite stray peak @ 230.7
Also the slope way from control from 270-300 nm

I think you do have something. Keep at it.

There is one thing that I would like to do tonight. Is there any reaction between blood and the TSP?

Some very interesting and intriguing effects have taken place within the observations:

1. The TSP actually destroys the nucleus effect, quite surprising.

2. However, the TSP also immediately damages and warps, contorts, flattens & destroys a portion of the red blood cells.

3. The most intriguing observation is that the TSP seems to selectively destroy some cells but not others. I have no idea what might be the agent(s) selecting cells. It does not, at this point, seem to depend upon the existence of the CDB. Surprisingly the majority of the cells were relatively free of the CDB.
Very intriguing here.

The question of why the coucleaux was broken up is also very intriguing - what is the mechanism and is there anything that can be identified to be of advantage here???

There is another very obvious and pressing question now - what is the effect of the enzyme treated TSP upon the blood.

Sep 12 2023

Repeat UV scan of TSP01 w/ large enzyme
Already result clearly to show themselves

TSP01 Control: Peak @ 272.3 well defined
tyrosine peak develops @ 226

TSP01 w/ L enzyme Peak @ 271.5
and not as well defined with
a broader peak. Also slopes
diverge between 274 – 300 nm.

Also a well defined peak @ 227.6

Therefore we have 3 noticeable differences
between the TSP & TSP + L enzyme.

These changes are also consistent w/ the previous comparison
trials w/ the L enzyme. One of the longer
term trend changes also appear to be the flattening
of the curve 266 – 274 and it is expected
that the eventually encompasses 260 – 274.

This is all very encouraging.

We have two other important observations that also indicate protein impact:

1. Filament colonies did not form in the L enzyme vial but they did in the S enzyme vial

2. Crystal biology did not develop in either the L or S enzyme vials.

Therefore some pretty big things going on here.

1. Enzymes show impact
2. TSP impact upon blood - rouleaux reduction, selective cell destruction, enzyme effects
3. Strong film already forming on culture surface w/ starch addition to culture.
4. Avogadro modely coming into play

On the starch culture, there is absolutely a film layer forming. It is a CDB film-complex layer. We do see the additional microbial form emerging as in the potato culture. At this point the culture is not distorted & remains easy to assess.

It appears as though starch may indeed enhance the culture and provide for surface growth also.

Phosphoric acid should probably be added to the molecular model based upon previous work.

Quite fruitful work w/ the most recent completed culture. This one was not overheated and looks much more "lively" or more pure. The color is the same as the original culture, ie light green vs the darker yellow undoubtedly from excessive oxidation by overheating.

The precipitate protein is also settling out very well after treat adjusting the entire solution from original pH ~5.6 to now ~6.8. In general we have a less oxidized protein. Call this one TSP 03. This is not the next generation culture that has starch added to it.

Since we have doubled the production rate I can start the next one also. I will add starch based upon rapid growth of surface film. Two cultures will be in the wing.

The starch is obviously a very important addition. It allows for advanced growth form rapidly. The culture medium is

1. Sugar
2. Starch
3. SPONCH fertilizer S, P, N, Fe^{+2}

Total Nitrogen 10%

4.90% Ammoniacal Nitrogen

5.10% Urea Nitrogen

Phosphate P_2O_5 8%

Soluble Potash K_2O 8%

Calcium 3%

Magnesium 1%

Sulfur 5%

Iron 1.5% Ferrous Sulfate

Manganese 0.2%

Zinc .05%

4. Pinch Pink Salt (1/16 - 1/8 tsp)
5. ~ 1600 ml H_2O

Well, I seem to be back @ the point of producing artificial erythrocytes - seems hard to explain in any other fashion. Very rapid growth, culture is only 24 hrs old. Surface layer, CDB en masse, erythrocyte form (settled). 85-95°F.

Note This (Sep 25 2023)

Now what is fascinating here is that TSP01 selectively disintegrates fresh human blood erythrocytes, but here once again, they seem to be able to be produced w/in the culture medium. Science fiction supreme.

—

* I have added peroxide to the culture slide. These observations

1. The "erythrocytic forms do not seem to be seriously impacted.
2. What I presume are starch cells are in that they are turning purple
3. But most interesting, the CDB protein cluster seem to be turning a vivid green color.

Iron + Magnesium → green

—

My balm: methyl salicylate, menthol, tea tree oil + Creosote bush powder (chappard) definitely brings this material to the top of the skin. Ultrasound degrades it in return (with some pain that is required in the process of healing). It is a slow, if not a continuous haul to pull this from the body.

There is most definitely a reaction between the culture medium and hydrogen peroxide. Catalase? What part of the culture is generating the reaction?

CEC Blood Observations - CDB saturation seems to be declined relative to Aug 18 inspection. However, complete rouleaux still occurs on bare blood slide prep.

TSP 01 + L Enzyme leads to same selective blood cell deformation, however rouleaux is eliminated. Same as is the case w/out the L enzyme added.

An important part of our UV enzyme testing is adequate controls. I have done it once, but it needs to be confirmed that the ENZYME ALONE in H_2O does not produce the same profile as post mixing TSP w/ the enzyme.

This is a critical step. In a sense, two controls. One the TSP alone, the other the enzyme alone.

Let's characterize our TSP03
pH we know ~ 6.0
Conductivity 16.61 (about the same as blood should be)
TDS 8851

Scan 1 TSP03 300 µl in cuvette
Scan 2 TSP03 + L-Enzyme 300 µl deluted 200 µl

Well we have our answer. There is a radical alteration of the protein, in the Case TSP03 from before. The two profile are not even close and that is what I remembered from before

First, I had the enzyme concentration way too strong, probably by a factor of 100 or more. Then were major disturbance taking place. I then took a first 5 ml sample of TSP and then took only 200 µl from the original L-enzyme solution (also w/ TSP03) and mixed that. O may a deletion.

The profiles are not even close.
The enzymes are having a significant effect.
Now you need to know which enzymes (s).

The trial is a complete and total success.

I suspect the TSP03 buffer itself made a big difference as there has been no time allowed for crystal formation. Also the color (light green) tells us the protein was prepared more favorably.

Superb work.

Three reasons we know the enzyme had effect:

1. No filament colonies
2. ~~No~~ Crystallization
3. UV profile completely different

] TSP03 + L. Enzyme

Sep 13 2023

Hey

The lab is looking really really good now. Except for Mid IR, you have duplicated much of the original lab capability in 15% of the space. Mid IR & Gas Chromatography & fume hood are the main items lacking - you can certainly do good work here.

Chemicals for qualitative testing is also a much spot that sucks away when I have put together

1. Incubation + Evaporation
2. Testing in all main practical manuals
3. Centrifuge
4. Binocular Scope
5. Culturing
6. Titration
7. UV-VIS-Overlap NIR spectrometry
8. Column Chromatography LPC-HPLC
9. Refractometry
10. Electromagnetics - freq generation (programmable) & oscilloscope
11. Potable VIS spectrometry
12. Ultrasound imager
13. Ultrasound generation
14. NIR
15. Electrochemistry - Inorganic Analysis
16. High level Microscopy
17. Education course
18. CO_2 & Air Quality Monitoring
19. MIDIR Vacuum Library
20. USB Microscope
21. Numerous small instruments

22. In Counter - absolute
23. EMF Meter
24. RGB Spectroscopy
25. Reflectance Spectroscopy - discrete
26. Cell Sensor - EMF - Gauss meter
27. Electrical Conductivity
28. pH
29. Refrigeration
30. Burette

14 Red & Blue Dyes
15. Tartaric Acid
16. Acetone
17. Acetone, Stripper, Thinner
18. Enzymes
19. Pink Salt
20. Sand

31. Oxygen Meter
32. Moisture meter

Chemicals:
1. Ammonia
2. Conc. Acetic Acid
3. H_2SO_4
4. Bleach
5. NaOH - KOH - NaOH
6. Methyl Salicylate
7. $CuSO_4$
6. Powdered Milk
7. Fluorescein
8. Sugar - Starch
9. Sulfur
10. $FeSO_4$
11. Baby Powder
12. H_2O_2
13. Isopropyl Alcohol