The Methylene blue story

What if you want to start your own garden of cheesy bacteria?

Mannitol salt agar is really salty, and this selects for staphylococcus, and micrococcus. Does sweaty feet and the salt therein help select for gram positive cocci? Good question. 10% Sodium Chloride is pretty friggin salty. 0.9% NaCl is just slightly hypertonic to us humans. 5-10% salt is pretty hard to imagine in armpit or feet.

Sweet aside: osmotic diarrhea and party smoke/fog

Mannitol is one of those short poly-alcohols that taste sweet. Glycerol, sorbitol, xylitol etc. We can make use of glycerol, but the others aren't readily absorbed in the gut, and so they find use in sugar free gums. Sorbitol is used to sweeten Pepto-bismol. A replacement for sugar? No, they cause osmotic diarrhea/laxative effect if consumed in any appreciable amount, and there are microbes in the gut can ferment each of them. Mannitol finds its way in the hospital as an osmotic, reducing extravascular fluid volume, reducing swelling in the brain and other extravascular compartments. PEG poly ethylene glycol taste sweet in a sickly sort of way, and is consumed as a laxative, or prep for colonoscopy procedures. You can spray PEG solutions on a hotplate to generate quite a bit of smoke. This is the juice they use in "fog machines" Glycerine works just as well, and smells a bit sweeter, but is more expensive source of "fog". As far as osmotic laxatives go, Magnesium salts have traditionally been used in this role, as Mg isn't readily absorbed from the gut either, but is unpalatable in its own way. MIlk of magnesia (magnesium hydroxide/"Maalox") can buffer your heartburn as well as getting your colon full of water. To increase its palatability, sorbitol is added.

Mannitol salt agar. Fermenters of mannitol like Staph Aureus turn the reddish pH indicator yellow The other not-so-pathogenic Staph, don't seem to be mannitol fermenters, red colonies from the reddish agar.

Polymyxin B sulphate, and neomycin sulphate are often combined in growth media selective for streptococcus. This happens to be two out of the three ingredients in Neosporin. The missing actor? bacitracin. Interesting.

Phenylethyl alcohol agar is a cheap, selective growth media for gram (+) microbes, streptococcus. The gram (-) just can't seem to make their cell walls/polysaccharide coatings in this stuff.

How about the corynebacteria? They are the key stinky cheese/foot microbe here, no?

There is this weird tinsdale agar, or tellurite agar that will promote the growth of corynebacteria as well as a few other gram (+), but the corynebacteria form brown halos around the colonies. Tellurium (from tellurite) is one of those periodic elements, we never talk about.

Enough of that. Onto the sneaky point of this lecture.

Rehashing the past: The blue bottle demonstration. Methylene blue

You may remember. long ago I did a demonstration with methylene blue. A soda bottle almost filled with water, a pinch of methylene blue powder, or a squirt of the methylene blue you can get for your fish tank, some glucose, and sodium hydroxide. When all three ingredients were mixed in my soda bottle of water, you had a dark blue solution that gradually became colorless. Put the cap on, and vigorous shaking brought the blue back, only to fade again. One of the very first chemistry demonstrations I did for Mesh. From the Bassam Shakhashiri books.

Oxygen. Redox. In the alkaline environment, reduction is promoted, lots of electrons around. and the reducing sugar glucose, reduced the methylene blue to the colorless leucomethylene blue. At the time, I had to get the glucose in China town (Glucolin), and pure lye was available as "Red Devil" lye. No longer available, but pure lye can be found in the not-so-brand name drain openers. Most drain cleaners have other garbage (excipients). I claimed all the ingredients could be found by the student. Fructose is a reducing sugar as well, and easily found in corn syrup, but I never tried it, preferring a powdered sugar, for convenience and consistency with story telling. Its easy to find glucose in other countries, The US? Not so much. Hence the Glucolin from chinatown. I blame the big corn conspiracy.

Story? How did I remember that chain of events? Because of the other glucose reduction stories. I didn't have the resources for it at the time, I really wanted to do the Silver nitrate mirror reaction. Tollens reagent. An ammonia laden solution of silver nitrate will precipitate pure silver against the wall of the container, forming a mirror, when glucose (or formaldehyde) is added. Beautiful stuff. Again described well, by Shakhashiri

Not as beautiful, but conceptually identical: Fehlings solution, benedict's reagent. Leave a copper penny in strong ammonia water for a long time (days), a blue solution will result. When reduced, pure copper will precipitate. The poor man's mirror demo. From Nathan Shalit's "Cup and Saucer chemistry"

The actual Fehlings has cream of tartar in it, tartaric acid salts, and the copper comes from copper sulfate, but the ammonia & a penny works too, and is easier to come by. Used to test for reducing sugars and for deciding if something is an aldehyde or a ketone. Aldehydes reduce, ketones don't do much without more reactive nudging. Toss a raisin (glucose) in there and copper stuff will precip out. Ta Da!

If you are bent on making Fehlings or Benedict's, you can get copper sulfate at Home depot or the hardware store. Its sold as a root killer for septic tanks. I guess you flush it down the toilet or pour it into the septic tank and it keeps tree roots from invading? Toss some copper sulfate on your campfire for some pretty green colors. Add a sodium/potassium free chlorine donor (like PVC plastic) for a beautiful blue flame. Burning PVC is a bit blech though.
You can use either reaction to test for glucose in urine.

Is it used in glucose urine test strips?

Good question. I think glucose oxidase is actually used in those strips. Extracted from yeast or fungi or the like. I couldn't tell you what the color reaction is.

Fehling's or Tollen's reagent useful in an austere environment? Dunno, probably not. Presumably you should be able to taste the glucose without doing the tests. And those test strips are probably more handy, stable, sensitive.

False positive results (for glucose) can be had when Vitamin C is in the urine. Ascorbate, an antioxidant, a reducing agent just like glucose and aldehydes

Anyways...methylene blue from the blue bottle demonstration. I told students they could find it at pet shops among the fish supplies. You add it to your fish tank to cure fishy diseases. ie. It has some antimicrobial activity. *Here we cross paths with the mannitol salt agar, the tinsdale tellurite, kryptonite whatever agar for corynebacteria.* SO What if you *don't* want the gram + cooties, and want only the gram negative, which are mostly associated with the innards of your guts and the sewer like openings at either end? Well, there is selective growth media for those guys as well. The microbiology folk are familiar with EMB agar (Eosin, methylene blue) will actually inhibit gram + growth and select for gram negatives of the enterobacter/coliform sort. Another example is Macconkey agar which contains crystal violet to inhibit the gram +. Ah ha. This is how we will distinguish our stinky feet bacteria.

MacConkey agar has crystal violet, bile salts (seems to prevent Gram + too), lactose +neutral red. Fermenters of lactose, like E.coli turn the agar pink.

EMB agar, lactose fermenters like E.coli, give iridescent dark green colonies(eosin at ph3.5+dark methylene blue) Eosin is present as a pH indicator.

The added value to our blue bottle demonstration: methylene blue (& crystal violet) inhibit Gram + growth. Will prevent stinky cheese and feet, and contains a lesson of biochemistry)

Why aren't these used as a topical antimicrobial for these gram (+) cooties, that camp out on our skin, make us stink, and make stinky cheese?

Dunno. I think the blue just makes a mess of things. Of course, there is gentian violet (crystal violet), an old and messy remedy for thrush (Candida) and a key component to the gram stain to differentiate between gram (+) and gram (-) bacteria. Hmmm, maybe it likes bacteria, just not the coated ones? (gram -). Come to think of it, another gram component is lugols iodine or grams iodine (more dilute). Hmm.

Both gentian violet and methylene blue appear to be making a comeback in some wound dressings, as MRSA becomes more problematic. There has been some work with using these dyes, to stain bacteria and make them more susceptible to photoinactivation, but I'm not really familiar with that stuff.

Gentian violet (crystal violet) is still sold in some places. In the old days, for nursing mothers with thrush. Coat the nipple, let the baby suckle. Baby gets treated, mommy gets treated.

Crystal violet is also useful for developing fingerprints off of the sticky side of tape. Pretty neat.

The blue bottle with milk?

The Methylene blue reductase test is an old assay to determine if milk has spoiled prior to where it is obviously sour, curdled. A certain amount of methylene blue is added to a certain amount of milk, and you wait. If the blue disappears in a short time, you got some bad milk. If it takes forever for the blue to be reduced to the colorless leucomethylene blue? Milk is good to go.
Good for an austere environment? Dunno. It has it's caveats. Its more of a quick-n-dirty assay. The results you get don't necessarily jive with the total amount of active bacteria present. The bacteria deplete the oxygen available in the milk, and the reduction of methylene blue starts from there.

Will shaking the milk like the blue bottle demo, bring the blue back?

Yes it will. And yet you still have the same number of bugs in there. Only the oxygen content has changed.

Another connection with the past:
Methylene blue is a phenothiazine (see Drugs and Human Behavior-Tibor Palfai) just like chlorpromazine (Thorazine) and promethazine (phenergan). Crystal violet, cresyl violet and thionine too! Both have antihistamine activity. Does methylene blue? Good question. The

anticholinergic effects of both also reduce nausea and vomiting, and generate complaints of dry mouth etc. (a la atropine) Somehow these anti-emetics have been replaced by Zofran. Dunno why.

I told the story of how chlorpromazine was the first neuroleptic drug. In 1953 used to treat a woman with schizophrenia. How its effects and side effects resulted from the promiscuous interaction it had with various receptor types Dopamine, acetylcholine, histamine, serotonin...

And it would appear, that our blue bottle methylene blue, is just as promiscuous.

Sneaky biochemistry lessons: The blue bottle kills malaria and reverses methemoglobin? Creates methemoglobin?

The antimicrobial activities of methylene blue extend even further. Paul Ehrlich (of Microbe Hunters and Salvarsan 606 fame-the original "Magic Bullet") noticed it killed/inhibited the development of malaria. This shaped his initial thoughts on how the immune system worked. I'm not really sure why methylene blue didn't have widespread use in the treatment of malaria, but I'm guessing the quinine based drugs came along about the same time, and were probably easier and cheaper to produce. Quinine literally grows on trees, well within them.

Can't find methylene blue in the pet shop? Well, in the clinical setting, it's used intravenously to reverse methemoglobinemia. Reverses methemoglobinemia? How fortuitous! Perhaps you tried your hand at amateur dentistry, armed with the info from last lecture. You used chlorinated brake cleaner (dichloromethane) to induce anesthesia, and have noticed your patients are listless, and cyanotic. You gave them methemoglobinemia. It happens more often than you think. No worries, the redox powers of methylene blue can reverse this.

Methemoglobin, in aggregate, is just the oxidation of the iron in the heme hemoglobin to a more useless state Fe3+ (ferric) . Oxidation=more positive. So it has to be Fe2+ to Fe3+. Carbon monoxide can accomplish this. Its nitrogen cousin, nitric oxide too. As well as a short list of other substances (or their metabolites) that can directly oxidize the hemoglobin iron. eg. benzocaine, dapsone. Methylene blue too! (in larger doses or in certain individuals)

Too much methylene blue can cause methemoglobinemia, especially in some individuals. Glucose 6 phosphate dehydrogenase deficiency.

Remember the pentose phosphate shunt? Well, neither do I. Regardless, its a process parallel to the goings on of glycolysis, but it takes the 3-carbon phosphorylated thingies and directs

them towards the manufacture of 5 carbon sugars. Pentoses. Ribose. The stuff of nucleotides. Stuff you need to make more DNA and RNA. Downstream from this, you involve folate in purine synthesis, and start talking about methotrexate and the like.

Upstream from the pentose sugar stuff, NADP+ is reduced to NADPH, and this supply of NADPH keeps glutathione in a reduced state. These two reducing equivalents (NADPH and glutathione) are needed to keep reactive oxygen species and radicals under control. Its an important function of the pentose phosphate shunt.

When methylene blue is delivered to the red blood cell, it is reduced (becomes more negative) via NADPH and an enzyme or two, to form the colorless leucomethylene blue. Every leucomethylene blue molecule made, depletes one NADPH, regenerating NADP+. Leucomethylene blue, in turn, can reduce methemoglobin, donating electrons, converting the Fe3+ to Fe2+. Reversing an acquired methemoglobinemia. Ta da!

If given in large doses, methylene blue can deplete most of the NADPH, leaving the RBC is a sad state, prone to oxidation, and in turn promote the formation of methemoglobin. Oops. And yet, it is believed that this NADPH depletion, and the resulting oxidative environment, is what makes life difficult for the malaria trophozoite. Hmmm.

I would appear that evolution has tried this anti-malaria strategy itself. Discovered it by coming along along with methylene blue and other anti-malarials and, in some people, managing to push things to an extreme. Acute hemolytic anemia. Oops

GLUCOSE 6 PHOSPHATE DEHYDROGENASE + GLUTATHIONE REDUCTASE, g6pd deficient people.

Individuals deficient in Glucose-6-phosphate dehydrogenase cannot generate NADPH from NADP+ at the same rate as others. Glucose-6-phosphate dehydrogenase is the enzyme that catalyzes this generation of NADPH from NADP+. It turns out that G6PD deficiency is an x-linked mutation, so boys are more prone to the phenotype. Heterozygous girls? I dunno, how this would present in heterozygous individuals. Through x-inactivation, you'd expect half of their RBCs deficient.

When faced with an oxidative stress, levels of NADPH decline, get exhausted, as well as levels of the downstream glutathione that depends upon a supply of NADPH to stay reduced. Worrisome reactive oxygen species (ROS) get to cause damage when reducing equivalents are in short supply.

This worrisome state is worst within the red blood cell. The poor RBC. No nucleus. No mitochondria. No machinery for transcription, translation, protein synthesis. No dynamic way to adapt. Only in us mammals do the baby RBCs, the reticulocytes, eject their nucleus into the great beyond. Other cells of the body could cope by conjuring up some bandaid proteins in reaction to this stress, but that requires a nucleus, and organelles. The RBC has none. The fact that it's loaded with hemoglobin, and more pointedly, Iron, makes the need for oxidation/reduction management much more important. Heme sets things up for oxidation, all day long. Remember cytochrome p450? Heme in there. Cytochrome C (oxidase) from the electron transport chain? Heme in there too. Methemoglobin and oxidative damage is a constant concern in the poor RBC.

Heme is a problem for the malaria parasite too! The parasite gobbles up the rest of the hemoglobin protein as food, but finds the heme toxic. All that oxidation. Through a complicated bit of biochemical gymnastics, the malaria parasite manages to get the free heme to stack up and crystalize/precipitate into hematin and hemozoin pigments. Sabotaging this malarial sabotage of heme is one mechanism by which some antimalarials function.

aside:
I'm embarrassed to say, I did not know this, (mammals + anucleate RBCs) until I looked at a slide of Emu brain I had prepared for someone at the Harvard Field station out in Concord MA. They did old fashioned physiology out there. They had Emus jogging on treadmills. How the technicians got then on the treadmills? I dunno. One died, and its brain found its way to me.

I saw these flying saucer parasite things everywhere in all of the blood vessels and was WTF are these monsters? Then the vet told me. Birds, fish etc have nucleated red blood cells. I was a mammalian physiology bigot. How embarrassing.

A common theme: Overwhelming the reducing mechanisms of the RBC, oxidative stress in the RBC causes chemical chaos, eventually sulfhydryl groups on the hemoglobin chains oxidize, condensing into Heinz bodies and a confusing array of hematin pigments. Damaging the cell membrane and initiating eryptosis (RBC apoptosis) and the RBCs lyse, spilling their hemoglobin laden cytoplasmic guts into the bloodstream compartment. Hemolytic anemia. You lose RBCs, all that hemoglobin converts to bile pigments, causing jaundice etc.....a story for another day.

In the glucose 6 phosphate dehydrogenase deficient person, NADPH and glutathione are lacking, but for the most part, are adequate, and the person is none the wiser, asymptomatic, their RBCs chug along doing their thing.

But.

BUT

Movie quote, Hannibal Lecter said: “A census taker once tried to test me. I ate his liver with some fava beans and a nice chianti. “

But favism (oxidative stress from a bean) or methylene blue or...

Fava beans upset the applecart in most, but not all of these G6PD deficient individuals. Fava beans contain some poorly described substances that oxidize hemoglobin, and that's bad for the G6PD deficient individual, their RBCs are just barely keeping oxidation under control. With additional oxidative stress, methemoglobinemia and frank hemolysis of the RBCs can occur. Not good. A different flavor of "favism" can occur , especially in G6PD deficient individuals, when Methylene blue does it's thing and reduces amounts of free NADPH and glutathione, leading to methemoglobinemia and hemolytic anemia rather than preventing it. Systemic infection also depletes reducing equivalents, and can lead to hemolytic crisis in the G6PD deficient individual. Dapsone and chloroquine can produce hemolytic anemia in G6PD deficient individuals via a metabolite that oxidizes hemoglobin. G6PD deficiency was first described in patients who developed hemolytic anemia after receiving dapsone and chloroquine. Benzocaine seems to precipitate methemoglobinemia in a few people, especially when exposed to it via endoscopy.

This sabotage of reducing equivalents may very well be the mechanism by which methylene blue inhibits malarial development. The malaria parasite within infected RBCs depend on the pentose phosphate shunt.

Did Hannibal Lecter get the favism? I don't think so. The glucose-6-phosphate dehydrogenase deficiency mutation seems to occur in regions where malaria is endemic. Lecter was too Northern European.

And just like sickle cell anemia, these folk are wee bit more resistant to malaria than others. Evolution? useful?

Connections: Malaria Its why people try to catch mosquitoes with cheese that smells like feet. The blue bottle demonstration. EMB agar inhibits gram + bacteria. Treating malaria with methylene blue. People from malaria endemic regions, carrying a mutant gene, that give some resistance to malaria infection,while simultaneously making them susceptible to a drug induced (methylene blue) hemolytic anemia. Such individuals are also prone to methemoglobinemia. They lack reduction.

Methylene blue consumes NADPH (the product of the enzyme, glucose-6-phosphate dehydrogenase, the first step in the pentose phosphate shunt) to become leucomethylene blue. Leucomethylene blue can reduce methemoglobin, regenerating useful hemoglobin as well as being antimalarial. In G6PD deficient individuals, methylene blue may create a an RBC crisis when there is no NADPH to spare. Glucose-6-phosphate dehydrogenase deficiency may be an evolutionary attempt to produce this "methylene blue" antimalarial effect, preemptively.

Are they more susceptible to the hepatotoxicity of chloroform and dichloromethane?
Yes. Halogenated hydrocarbons will deplete hepatic glutathione in the healthiest of people. G6PD deficient? Oh yeah.

Could you reverse methemoglobinemia with ascorbate?

What?

Fehlings reagent to test for glycosuria. You said vitamin C in the urine would make the test "positive for glucose" A reducing agent/anti-oxidant performing the reduction glucose would have done. Instead of methylene blue, would vitamin C work?

Good question. Hmmm...

Good news. Vasoplegic syndrome.

Profound hypotension that you see in states of sepsis, large burns, anaphylaxis, and in the period after heart bypass surgery. That heart bypass perfusion pump may seem like a passive actor, pumping blood, oxygenating it, etc. but it does job on you, your blood, and your immune system.

That does not sound like good news.

I'm getting to that.

In 1992, before Mesh, nitric oxide was the newest neurotransmitter, and the first gas to have said job description. Exciting neuroscience stuff, but in the decades that followed, nitric oxide has become the rock star of endothelial dysfunction, that poorly understood component of Virchow's triad of thrombosis. The endothelial cell would tell us much about some of the most frustrating disease states with the highest of mortality. A story for another day.

After surgeries involving heart bypass, or anaphylaxis, burns, sepsis, some patients would develop a profound hypotension, that customary vasopressors couldn't touch. Vasoplegia. Epinephrine, dopamine, vasopressin did not work. Methylene blue did. ie.**The Good news**

It turns out that in addition to being a functional inhibitor of glucose-6-phosphate dehydrogenase (it sucks up NADPH when giving in large doses) it inhibits Nitric oxide synthase, and guanylate cyclase (a GTP-cGMP second messenger)

Promiscuous indeed.

Its interesting that vasopressin/ADH is an arginine containing peptide. Nitric oxide synthase converts arginine to citrulline. Hmmm? Maybe not so hmm.

Another clinical connection to the "good news": Sickle cell disease

Sickle cells, and hemolytic anemia, jaundice etc. Sickle cell trait can predispose you to acute hemolytic anemia, in ways that are very reminiscent of the above mentioned mechanisms. Its believed that the free hemoglobin released, scavenges NO, nitric oxide, contributing to a chronic pulmonary hypertension. Sickled cells tend to clog capillaries in the periphery and create a lot of vascular problems for the spleen. Also priapism?

Priapism. Back in the day, I'd talk about men hung from the gallows, priapism and myth of the mandrake plant. Today? You know the viagra commercials that caution you to see your doctor if you have an erection lasting more than four hours?

That's priapism. It's no joking matter.

Incidentally, 4 hours is about the same amount of time you can keep a tourniquet on a limb, before irreversible injury and cell death occurs. Hmmm.

The sickle disease patient, often children will suffer from sickle cell disease induced priapism.

And?

You can treat this with methylene blue. In addition to nitric oxide synthase, methylene blue inhibits guanylate cyclase. Promiscuous!

Viagra, Cialis, inhibit Phosphodiesterase 5 (PDE5) which ordinarily degrades the cGMP generated from active guanylate cyclase. The simplified model: Nitric oxide synthase produces NO, NO signals guanylate cyclase-->forms cGMP--->venous dilation-->erection.

Methylene blue inhibits both nitric oxide synthase AND guanylate cyclase.=no erection?

It will kill your boner, kids.

Added value! And for the individual with sickle cell disease associated priapism, methylene blue could be another tool in the tool box.

Connections:

Stinky feet and stinky cheese to trap malaria mosquitos? The corynebacterium stinky foot/cheese connection. The unexpected value of antifungal foot powder--anti stink, due to anti-gram+ miconazole. The not so awesome cyp3a4 interactions with imidazoles and Seldane. long QT interval. Something you get with antimalarials. Hmmm

Returning to the stinky feet and cheese bacteria. How to grow them in your stinky garden? Selective media. Interestingly, media selective for gram negatives contain methylene blue and its cousin, crystal violet. One of my first demonstrations! The blue bottle. More added value. These compounds can combat the bacteria of stinky feet and stinky cheese and MRSA with the super added value of being antimalarial, and an antidote for methemoglobinemia. The unfortunate but interesting finding that glucose 6 phosphate dehydrogenase deficient people are somewhat resistant to malaria, prone to hemolytic anemia, methemoglobinemia. Induced by methylene blue's inhibition of NADPH via glucose 6 phosphate dehydrogenase. Interestingly, all this hemolytic anemia and supposed protection from malaria infection sounds a little like sickle cell trait.

Drugs commonly associated with acquired hemolytic anemia? ...antimalarials. Chloroquine, dapsone.

Methylene blue can help treat malaria, sickle cell priapism, vasoplegic hypotension due to sepsis, anaphylaxis, burns.

Eating stinky cheese while taking methylene blue can cause tyramine cheese syndrome! Hypertension. Methylene blue inhibiting MAO as well. Serotonin syndrome!

One last rehash:

Remember the cheese syndrome story? The 2015 version of that tale would now include methylene blue. Methylene blue inhibits MAO monoamine oxidase! Yup. Promiscuous molecule it is.

Tyramine induced hypertension in people on MAO inhibitors. More recently, serotonin syndrome has been induced in people taking SSRI medications or with active carcinoid tumors. MAO promptly degrades serotonin, unless methylene blue gets in the way. Oops.

It turns out that the list of things that interact with monoamine oxidase is huge because a hell of a lot of biological molecules are...monoamines.

Don't eat that cheese?

Don't have that beer?

Wait, that sounds like someone on isoniazid (INH), the "original" MAO inhibitor. The apocryphal tale of tuberculosis patients getting serendipitous treatment for depression from their TB drug, INH. etc. A Story for another day. Tricyclics.

The blue bottle demonstration has much to say, as do the stinky feet and cheese. This is how I view the world, and what I hoped to share. Stuff you already know/knew just connected in a different way.