# HEMOLYTIC ANEMIA IN BLACK RHINOCEROS

**Don Paglia**

**TAPE 8B 135**

**Paglia**: I am essentially going to try today to summarize what is almost eight years of work. There were a lot of gaps in that time, because we did not have the sample availability that we have been getting recently. I am going to do somethings that you will not like, I am going to try teach you a little biochemistry; but I am going to do it as a surgical pathologist who hates biochemistry. How I found myself in this field is beyond me. I take a fairly simplistic approach to it, so you are not going to see massive 150 diagrams of metabolic reactions, but they will be a little symbolic. I am also going to present to you several human diseases and try to discuss some of the clinical and metabolic features of those, because of the parallels that exist between the rhino situation and those specific diseases. In a way, that is not an especially gratifying kind of thing to do, because as it has turned out, we have been sort of closing the circle on all of the animals that have contributed to understanding human disease. I think this is one of the few opportunities I have seen, and certainly the only opportunity I have had, to participate in something that allowed what we have learned about human disease to come back to the animals. I kind of like that.

In 1984 I got a call from Eric [Miller]. Eric had stumbled across some of our work over at *Barne's* Hospital Biomedical Library, if I recall correctly, in investigating his first case of hemolytic anemia. He started looking up hemolytic disease in humans, and ran across a few of our papers, and called up with a very very possible story. I of course violated the cardinal rule and gave him a diagnosis on the phone. I said that has to be G-6-PD deficiency, that is a classical story for it. It fit virtually every detail he presented. So I said, send us some blood and we will prove it. That started the odyssey.

Now I have to give you a little bit of background about the metabolism of red cells as we knew it up to the time, "BR"--before rhinos. If one looks at it very simplistically, there is virtually only one thing the mature red cell can do in terms of satisfying its energy requirements. It does have very specific energy requirements, despite the fact that its primary function of gas exchange is done without any energy requirement. In order to survive for whatever its finite life span is, in the peripheral circulation according to species--in humans 120 days or so, it has to fulfill certain energy requirements. It does that very very simply by taking glucose and degrading it to lactate. It can do that through two principal pathways.

The first one is the old classic Embden Meyerhof pathway of anaerobic glycolysis where it is broken down in 11 stages, and in the process generates two compounds that are important to the balance of energy within the cell. The one we know is the common 187 for all biochemical reactions, ATP. Beyond the reticulocyte stage, this is the only way virtually that ATP can be generated. So all of the metabolic energy that depends on ATP, the cation pump to keep electrochemical gradients in proper balance and all, all depend on ATP. Additionally, one of the pyridine cofactors that exists is cycled to the reduced state by

degradation of glucose through that pathway. This is the specific one for converting methemoglobin back to the functional oxygen form. So one of the *redox* reactions is also dependent on that.

A second pathway exists in which glucose instead of being degraded anaerobically, is oxidized. Carbon dioxide is given off, and the breakdown products can come back into this pathway and continue down. In the process a different pyridine cofactor is cycled to the reduced state, and this is in the electron exchange with glutathione, which we will get into a little more detail. That is sort of a fundamental reaction for neutralizing oxidant stresses. We can spend a lot of time on that because I think that is where our common denominators...**(TAPE FADE OUT)**

As I said, as a surgical pathologist I have to look at this simplistically, so I can not even remember the names of the enzymes, so I have eliminated this. But each of these sort of signifies one of the catalyzed steps in the Embden-Meyerhof pathway. The crucial thing I want to look initially is the first level of glucose is phosphorylated in the sixth position, and that is catalyzed by hexokinase. This is the pivotal compound. This can either be continued to be degraded anaerobically, or it can be that one that gets converted through the hexose monophosphate shunt. This is the G-6-P of that first reaction, glucose 6 phosphate dehydrogenase, which converts this substrate through here and cycles that pyridine cofactor, and maintains a pool of reduced glutathione. So just to reiterate, degradation of glucose anaerobically produces a pool of ATP, and that is the fuel for all of these specific reactions on which the normal life span and function of the mature red cell depends.

**Sadler**: Where are those taking place, these 216 in the red blood cell, are these all cytosol?

**Paglia**: Almost all cytosol, but some are membrane bound in whole or in part. Transport mechanisms for glucose is the facilitated transport, but once it is inside, most of that is cytosolic.

This shows a little more detail about what happens in the hexose monophosphate shunt. The two terms you will keep hearing implied are hexose monophosphate shunt or pentose phosphate pathway, because one of these carbons is given off and you end up with a pentose coming back in here; all referring to this oxidative pathway of glycolysis. During this process, the dehydrogenase reaction, and there are two of them, maintain a pool of pyridine called old TPNH, the pyridine cofactor in the reduced form. This is the hydrogen donor for taking oxidized glutathione back to the reduced form. We really ought to be talking about it from the other way, because physiologically and pathologically, what happens in an extremely broad array of different processes is that oxidants are produced. I have sort of shown a generic oxygen species with that particular symbol. Inflammation does it, breakdown of foodstuffs does it, infection, radiation, all of these things are mediated by the production of oxidants which have to be neutralized, otherwise essential cellular components are themselves oxidized, some irreversibly denatured. That is the mechanism or common pathway for *oxidant injury*.

When an oxidant occurs, because of the very low oxidation potential of reduced glutathiones, a simple tripeptide with a *sulfhydro* group, this is preferentially oxidized before other reactive *sulfhydro*

groups are, so we have a pole of a sacrificial reductive that is there, and says take me, save everybody else. It gets oxidized to a disulfide and could be all used up if this mechanism were not available to re-reduce it back to this form and allow it to sop up more oxygen. So if there was a sudden surge of oxidants for whatever reason, infection, inflammation, or stress, the tripeptide gets oxidized to a disulfide. This stimulates the re-reduction drop in this ratio stimulates glucose, increased metabolism of glucose and so more glucose will come in and produce more of this and an acceleration of glucose degradation through this pathway will occur. Under normal circumstances perhaps only 10% of the glucose that is being *eaten* up on a routine basis is diverted through this pathway. Under the stimulation of an oxidant stress, that can go up to 10, 20 or maybe even 30 times the amount. So the diversion through here is mediated by the ratio of these two compounds in their reduced and oxidized forms. It is a system that is nicely tuned to be stimulated in response to a sudden surge in oxidant stress. That is probably all the biochemistry we need to talk about.

What happens in human diseases and in a lot of other animal models with this *MG*, with this pyruvate kinase deficiency, the english springer with phosphofructokinase deficiency, PK is one of the last reactions down here, phosphofructokinase a little further up. But in general, if there is a defective enzyme in the Embden-Meyerhof Pathway, then the consequences of a blockade of glucose degradation, a lack of the production of adequate ATP, the concentration of ATP falls. The clinical consequence of that is generally the chronic hemolytic process. The cells do not live their normal life span, the marrow can make up for it, and you get a compensated anemia. If it does not, you will get true anemia. This is the classic pattern that we see with defects of this particular category.

By contrast, and again I am speaking in generalities, the defect in the shunt pathway, a glucose 6 dehydrogenase deficiency, results in a fall in the reduced form of pyridine cofactor, a change in its ratio, and a decrease in the amount of reduced glutathione. So these cells are less responsive to a sudden oxidant surge. They can not divert a large amount of glucose through here rapidly in order to cycle the reduced glutathione pool and neutralize a sudden surge of oxidants. So the clinical consequence then, is that when confronted with oxidants, the glutathione will fall. It will form as a consequence of the oxidation of the *sulfhydro* units, mixed disulfides with hemoglobin and with other membrane proteins. The consequence of that is Heinz body formations. Those are hard concretions that make the cell less deformable, so they are likely to be picked up in the spleen which has a slow stagnant circulation where the cells have to squeeze through where it is acidotic and increases glucose degradation, and where it is hypoxic. So you get hemolysis that will occur for as long as this situation exists. Often it is in response to any number of initiating events. It can happen with infections. It can happen with a lot of drugs, and indeed it was discovered because of the effect of the anti-malarial drugs on soldiers who where in malarial infested areas. Certain compounds 295 and some of those that are used against malaria, induced hemolytic episodes in people in whom it turned out that G-6-PD was in one way or another defective. Running into certain oxidant chemicals is another one. And classically, patients who have this deficiency--a small subset of

them, are very sensitive to some of the compounds, some of the plant metabolites in fava beans, which is the broad bean, a staple in the 302 Mediterranean area in many diets. All patients that are sensitive to fava beans, who have an acute hemolytic episode after consuming fava beans, or anecdotally, running through a field of blooming fava beans and inhaling something, have this defect. But all people who have this defect are not sensitive to fava beans. So there are a lot of things we still do not know about this. And additionally, it can be severe enough so that chronic hemolysis does occur. You see the two different clinical situations. The defect in this [Embden-Meyerhof]] pathway generally produces lower ATP and chronic hemolytic anemia. Whereas a defect in this [pentose phosphate] pathway may not be evident at all until a sudden challenge by oxidants occurs. When that happens, they can not respond by increasing glucose metabolism and diverting it through this pathway. So they will have an acute episode, which may be very self limited, because when those cells die, and apparently it is the older ones that do, the response of the marrow is to kick out young erythrocytes and reticulocytes. Those cells can work on oxidated metabolism to make their ATP, so the episode stops. As soon as the inciting agent is removed, there is no more need for that. So that is the classic pictures that we see in those two episodes.

So after what Eric [Miller] has already told you and what you know about the hemolytic disease in the rhinos, you can understand why I responded on the phone as I did. Eric [Miller] did send me some blood and this was the first patient that we saw, and we got our first look at rhino red cells. When we took our first look at rhino red cells, we simply did not have any idea what we would encounter. Obviously we were looking for a specific enzyme defect comparable to G-6-PD deficiency, or PK deficiency, or something like that, that would explain why these cells in animals that hemolyzed were sensitive and others were not. We expected there to be some single defective enzyme that would be compatible with all we knew about enzymopathies in humans and in other animals.

We of course needed to establish what were the normal values for a number of enzymes. This is not quite as formidable as it looks. What I have done here is just list all of the actual enzymes of the Embden-Meyerhof Pathway through here and a few ancillary ones. I related them to what we find in humans and generally in most other animals. Most mammalian species do not differ that much from human. So I have taken a human value regardless of what it is, as "one." So if a rhino red cell had exactly the same activities... Recognize that we are talking about a range of activities. Hexokinase in international units is about 0.25, triosephosphate isomerase may be 1,000 units. So we have got three or four orders of magnitude range in absolute units of activity in these enzymes. I have taken them all as "one" in humans and if the rhino were the same, then they would all look like aldolase. Aldolase is the only one that had the same absolute activity that humans do. In some instances, the activities were increased, and this is on a long scale, so about 25 times higher than human red cells is the hexokinase activity of a rhino red cell. The glutophosphate isomerase is up about 1.6 or 1.7 times the activity. Interestingly, most of the enzymes of anaerobic glycolysis were a lot less, and one of them, phosphofructokinase *could* qualify as a PFK deficiency. Here it is down to about a tenth of the activity that it occurs in human. When we see 10%

activity of any enzyme, that is an enzymopathy almost by definition. Now whether it is functionally significant, pathologically significant, is another matter. But this constitutes a pretty serious difference in comparison to what we have seen in other mammalian species.

Furthermore, there are couple of others that are worth pointing out. One of them for example, adenosine deaminase is the enzyme you have seen getting a lot of recent press, because this is the first correction deficiency of this enzyme in humans causes severe *combine* immunodeficiency disease, the hereditary counterpart, AIDS; and which had just become amenable to the first genetic engineering on therapy. This is a single isozyme in humans. It has tissue specific variation, but those are close translational modifications. Patients who are deficient in this in their red cells, are deficient in their other tissues, particularly their lymphocytes. They accumulate enormous amounts of deoxynucleotides, and they have a functional impairment in their immune system. We can not detect activity of this enzyme in rhino red cell. Nucleoside phosphorylase deficiency in humans is an equivalent one and its activity is less than a tenth of what we find in humans. So here are two enzymes that if anyone had come to us and said, we have this situation, we would ask them about the immune deficiency that exists. Now, are rhinos immune deficient? Is their propensity for fungal pneumonias have anything to do with it? Questions, no answers. But these are things to keep in mind when we are going back and forth into areas of commonality, and I think a number of them exist.

Interestingly, my prediction that they would have G-6-PD deficiency proved absolutely right, but in the wrong direction. It was not a deficiency, it was a hyperactivity. They had three to five times the G-6-PD activity in absolute units that we expected. Their glutathione peroxidase was about 13 to 15 times higher than humans. So we have a couple of instances where there is an accelerated increased activity rather than a deficiency.

These are the isolated enzymes. What I want to do now is go back to those original two pathways and give you a feeling for how this comes together if we look at a whole metabolic pathway, rather than the individual enzymes. Clearly, we are not going to find a single enzyme that is responsible for all of this. We have to start looking at the species characteristics and asking the question, is there something about the way rhinos have evolved their pathways of metabolism, that for them is normal, that gives them the propensity for these disease states? That is the attack that I am going to try and take here.

I have gone back to the basic pathway of anaerobic glycolysis. Again, regardless of how active, whether it is a thousand units of TPI or a couple of tenths for hexokinase, I have designated this as a circle of unit area to signify the absolute activity of each of these enzymes in human red cells. By comparison, this is the activity in the rhino red cells. These are black rhinos we are talking about exclusively until late in the game. Remember the 25 *whole* increase in hexokinase that catalyzes this first step reaction; and glucophosphate isomerase that will become important to you in a later consideration, is the only other one that has an activity that is higher than humans. All of the rest of these, including a couple of crucials that are considered rate limiting *reactos*, like the phosphofructokinase, the G-6-G-3-PD reaction, that in human

red cell metabolism are the crucial rate limiting reactions of glucose degradation by this pathway. These are very very low. So the overall pattern is one that seems to suppress the apparent need for generating what this pathway does, ATP. A very peculiar situation, unknown is far as we can tell in any other species.

We measured the ATP. And again, this is a very consistent finding among different mammalian species. The adenine nucleotides are almost all in the triphosphate form, there is a little bit of the diphosphate, and very little of the monophosphate. The total adenine nucleotides in human red cells comes up to this level. When we looked at those same parameters in the rhino red cells, and looked again, and looked again, because we did not believe it, we kept finding them down in this 3% and 5% range. This is an early slide, and this is a very consistent finding. We were faced with this dilemma about how to explain how a cell can function with so little fuel. We have long assumed the traditional assumption that the mechanism of hemolysis in a number of circumstances, but certainly in the enzyme deficiency where the ATP is low, is that it runs out of fuel to fuel those things like the ATP ace cation pump, *water moves in the cell breaks,* that whole sequence. We would see that or expect that when our ATP levels fell below half of normal, or a third of normal, but when you are talking about 20%, these are levels far and above what the rhino has, yet the rhino is existing in the ambient state with this amount of fuel. This is not rocket scientist stuff, this is aeronautical engineering stuff, because this is what we call the metabolic equivalent of the bumblebee to the aeronautical engineer who looks at a bumblebee and says, that thing can not fly. We look at this cell and say, that thing can not exist. Metabolically it is dead. But it does, it goes on. I do not know how long it goes on, and I do not know if anyone here has ever had the opportunity to do an actual *chromian* survival, or maybe the life-span of a rhino red cell; but given their peripheral blood picture, I do not think their turnover rate is... They are not flooded with large macrocytes and surely they do not have reticulocytes...

**O'Ryan**: We have done this in wild apparently in healthy rhinos, and we find exactly the same thing. This was your zoo animal wasn't it? We found this in two wild rhinos that were caught and being translocated and handled for whatever reason. We tested the red bloods for ATP levels and they are exactly as those.

**Paglia**: Interestingly in the white rhinos that we have had an opportunity to look at, it is also extremely low. This seems to be a general rhinoceros characteristic.

**Stuart**: How would the fact that the point that the rhino can use volatile fatty acids as energy sources, like the C2, C3, C4 acids for energy, how does that differ from the human who essentially uses all glucose?

**Paglia**: It would have to come back in at the mitochondrial level and onward where fatty acids could get...

**Smith**: It does not have any effect because cattle... When you look at ruminants you see more like a human picture except for G-6-PD, in sheep and goats. You can not use those, the mature red cell can not use them.

**Paglia**: I am aware of that 508* so I would suspect that the earliest forms 510 mitochondria that use..

**Blumer**: Have you had the opportunity to look at any of the Asian rhinos, the *Sumatran rhino,* or 513?

**Paglia**: We had one Asian early on, and we were not measuring this at the time, so I do not know that. We had a total of nine or ten whites and we were only measuring this in the more recent one.

**Keitt**: Are you going to show us other nucleotides?

**Paglia**: Yes.

**Jessup**: You and I talked before, have you calculated or can you calculate energy charge from those levels?

**Paglia**: You can calculate it, because we do it for 522 the diphosphate monophosphate form, it still would be lower than I would expect. The ratio is... Well, you can tell from here, that...

**Jessup**: But it is not as bad as I was thinking when I first read some of the numbers, looking at those at least.

**Paglia**: Well, it gets real tough because the numbers and absolute values are so low, that it would be a little shaky in saying that this 10% increase in ADP changes the energy charge enormously when you are talking about such very very low value. So I do not have a lot of confidence in energy charges being indicative of anything.

Alan's [Keitt] question about other nucleotides is interesting. We have not been able to get good pyridine cofactor assays. Our assays are not accurate enough for that. Corky *Tinacka* and Charles 538 down at Harvard UCLA, do have a good system, and Mark Scott at the Oakland Children's Hospital has attempted to assay that for us. That gets particularly important and relative to catalase deficiency that we will talk about later on. They require extremely fresh specimens and that has been a problem, and they require very special handling. So I can not give you good data on the pyridine nucleotides. There is however an indication, we discovered this early on, that some other kinds of nucleotides exist in here besides the adenine nucleotides. They are shown by what appears as just a spectral analysis of an acid extract of the red cells themselves. Since virtually all of the nucleotides in most mammalian red cells are based on adenine, the absorption peak shown by this dotted line is highly specific with the peak at about 257.5 nm. A ratio of 260 nm to 290 nm was very characteristic of this fallout of that down slop. That is a signature for adenine. It does not matter whether it is adenosine or ATP or AMP, the adenine base is the absorbent.

When we did this with rhino red cells we got these kinds of shifts which do not look like an awful lot, but their absorption peaks are up in the ratio of 270 nm and 280 nm, that is an enormous amount of change in extract. This is indicative of some other nucleotide base that is present in these cells, which we attempted to identify chromatographically. It is probably a uridine containing compound, and it may be a cytidine containing compound, a mixture. There is no simple one. We have had experience in doing this because one of the deficiencies discovered in our laboratory was pyrimidine nucleotidase which clears pyrimidines by breaking them down and allowing them to *clear* on themselves. When that enzyme is defective, either by a heredity deficiency or by lead poisoning which specifically knocks that out and causes basophilic stifling and lead poisoning, that is from an increase in the amount of accumulated pyrimidine compounds and they have absorption spectra like this. So when we saw this, we expected to find some kind of pyrimidine nucleotidase deficiency, of course again, they proved us wrong. I still do not know what those compounds are exactly, but uridine compounds are high on the list of possibilities. So we do have something else to explain... It can not be explained simply by a nucleotidase problem, they have abundant nucleotidase activity.

So we are back now to looking at these two possible pathways. Where we had just compared the left and right of the rhino situation for the Embden-Meyerhof pathway, and saw that we have a lot of very very low activities for that pathway in the rhinos. Now we are going to look at the [hexose monophosphate] shunt and take each one of these steps with these enzymes being the crucial mediators for each of those and give them a unit value in terms of area there. Then do the same thing for the rhino red cells. We get now a feeling for the overall metabolic pathway and how that varies from human. We have to consider hexokinase the first step in the shunt, because without the substrate for the shunt, which is generated by hexokinase, you have got no shunt. So if we look at this in terms of hexokinase being the first step, we have got an enormous increase of activity there. In the second step of G-6-PD, we have a pretty significant increase in activity. Further down we have got a glutathione peroxidase with a very significant increase. Two enzymes in here, the 6-phosphogluconate dehydrogenase and the glutathione reductase are lower than normal. This looks like it might be a bottle neck in what otherwise would be a hyperactive pathway. It looks like the rhinos have attempted to evolve a pathway that has increased antioxidant potential, but with a couple of potential blockades in here. When we look at *only the* absolute activity, and that is a major flaw in all of what we have been doing. We are looking only at optimum *in vitro* activities as done by systems that have been devised to be optimum for human red cells. Now I do not know anything about the Michaelis constant for these enzymes yet. I do not know whether this [6 phosphogluconate dehydrogenase] is a low optimum activity, but it might have a ten fold lower Michaelis constant, a greater substrate affinity and its activity could be functionally a lot higher. We do not know things like that, because that means redoing all of the assays, and checking all of the other factors, pH optimum, cation requirements, etc., to make sure we have optimum systems for the rhino red cell. That would be the only way we could come about that.

**Smith**: Are you running them at pH optimum for those enzymes?

**Paglia**: For selective ones we have, yes.

**Smith**: But like for G-6-PD and 6-PD, do you run those?.

**Paglia**: We have not done that.

**Smith**: No, but what I am saying, is when you run the regular assay are running at the human optimum for that?

**Paglia**: We run at the human optimum. We have done a number of these selectively, not done a whole pH curve, but tried up and down and tried different buffers and things, a lot of cation changes. We have not done a whole program and do as we should do 650 for rhino red cells.

**Smith**: I think running them at the pH optimum is not necessarily... My philosophy is to run them at the pH that they are at, which should be around 7.2 probably.

**Paglia**: Which is what we did.

**Smith**: *Ernie* used to run his at 7.6 or 8.0 or something like that.

**Paglia**: I think 8.0

**Smith**: I asked him once why he did that, and he said they had that buffer.

**Jessup**: Don, when you have looked, have you seen any of them responding to any pH changes or anything that you have seen? You said you looked at a couple, have you noticed any increased activity, or have they tended to stay in the same general pattern?

**END TAPE 8B START TAPE 9A**

**Paglia**: We have not *shifted* any of the assays.

**Blumer**: In other species where some of these optimum conditions might have been determined, is there much variation?

**Paglia**: As far as variation, John [Harvey] could probably tell us better. He has looked at for example glutathione reductase in horses, and cats and a few other critters. They do have different pH optimum, they have different Michaelis constant.

**Harvey**: Right, I think that the classic example that Joe [Smith] addressed is that G-6-PD *sheep* look G-6-PD deficient. But the differences in what they respond to, inhibitors and stuff, is quite different.

**Paglia**: There is some enormous amount of species variations which we have not really addressed here. So again, I can not give you answers on it because this is not sound scientific data to give you the kind of answers you are looking for.

But can we get some *efferential* information? If we say that this suggests evolution towards a higher antioxidant capacity, it is interesting to note that two very crucial enzymes have extremely low or virtually undetectable activity in rhino red cells, and that is glutathione-S-transferase and catalase. Again, this is the black rhino. We will get to those actual reactions a little later. This suggested though, that we should look at some of the antioxidant metabolism. We have a clinical situation that says that this is a G-6-

PD deficiency, how far does that translate into how a patient with that deficiency would behave? Does the rhino red cell behave that way? Well, we subjected the rhino red cell to some of the classical screening tests that are used for G-6-PD, the ascorbate cyanide test, there are a whole array of them. They are nonspecific, but they all require an ability under oxidize challenge, to start metabolizing glucose and shove it through that shunt to cycle glutathione. So glutathione stability is a classical test of that deficiency. Methalene blue stimulation of that shunt is a classical test. When we did that with the rhino red cells, they all behaved as though they were G-6-PD deficient. This is just one example of measuring the amount of reduced glutathione in the cells over a period of time under challenge of by an *in vitro* oxidant, ascorbate. Here you can see it has a dose sensitivity, and a fairly uniform K rate that is very reproducible in rhino cells. This is about what normal human red cells would show with this challenge. This rate in here is what G-6-PD deficient red cells might show in terms of their glutathione stability. It does not matter whether the oxidant is ascorbate or *acetylfedrohydrozine*, we tried that and we tried peroxide, *tributal* hydrogen peroxide and things like that. The same kind of thing happened. They behaved as though they were G-6-PD deficient, but they had lots of G-6-PD.

**Smith**: In the 035 have you ran the glutathione down and then watched it come back?

**Paglia**: I have done that. Some of the studies that you have published on horses, GSA regeneration rates, inspired me to do precisely that. They will regenerate it right back up again. How? We can run it down all the way to the bottom and their regeneration rates are just like humans.

**Harvey**: I think that one of the dangers of the ascorbate screening test... I did it on three species, and two of the three dogs I think and horses both looked like G-6-PD deficient humans, turning the sample brown. I think one of the dangers when we are doing *in vitro* tests where you are blasting them with high doses of the stuff, is that certain things become limiting that might not become limiting in the natural state.

**Paglia**: That is true.

**Harvey**: It is almost the same problem we had with running enzymes at B-max when we run them with screening tests at B-max, stuff the speed the dogs as far as I can see are not normally susceptible to oxidants excessively. But on that screening test you would say, whoa boy, they are like they should be real problems like in humans. That is one of the concerns..

**Paglia**: We used the screening test as that, as a screening test, then stop right there; and then went to quantitative assays. We would vary the amount of the challenge oxidant, and if you do that you will find that they will crash with perhaps a tenth or less of the concentration of a given oxidant that would cause that in other samples.

**Harvey**: Now, did you add cyanide to any of these?

**Paglia**: No.

**Harvey**: Because with a low catalase they are going to be at a disadvantage compared to a human when you do those...

**Paglia**: You are right, but the cyanide ambition does not effect these. They are sensitive regardless of that, and sometimes ten fold more sensitive or more to a given concentration of a given oxidant.

Among things that occurred with them when challenged with oxidants, is that they form Heinz bodies in enormous numbers. We started looking at Heinz bodies with the shipment of ten that came in from Zimbabwe, doing regular Heinz body preps. We found that the animals, in all of them we looked at, normally had about 10% or 15% Heinz bodies in the peripheral blood. These are huge single very prominent Heinz bodies. If that is their ambient level, again it suggests that something is going on that is causing an oxidating denaturation of some of those proteins involved in hemoglobin and membrane compound that causes those to form. When they were challenged, that will go up to 90% or 95%.

**Harvey**: The one caution I would put there is that cats normally have 5% to 10% single large Heinz bodies, and they will go up dramatically with oxidant challenge because they have hemoglobin that has a lot of *sulfhydro* groups. The biggest reason I think cats have so many Heinz bodies is that they have a non-pitting spleen. On ultrastructure, their spleens have big gaps, and the whole cell can go through. So cats are one where splenectomy or nonsplenectomy does not seem to matter. Do rhinos have Howell-Jolly bodies or no?

**Paglia**: Not Howell-Jolly bodies.

**Harvey**: Because usually if it is a poor pitting spleen, I would think you might also have a bunch of Howell-Jolly bodies.

**Paglia**: I do not see those, and I do not know about the spleen. I think that is a very appropriate question.

**Keitt**: Some people are asking what are Heinz bodies and Howell-Jolly bodies..

**Paglia**: The Heinz body generally is a small glob of denatured protein attached to the membrane. It generally involves mixed disulfide formation with denatured hemoglobin, *sulfhydromine* oxidized generally with certain protein components of the membrane itself. They are generally attached to the membrane. They are like little pebbles in there, and when the cells squeeze through the spleen, these will either be pitted off and the cell will clear its Heinz body and move on, having a little less volume; or it will get hung up. If it gets hung up, since the spleen environment is very acidotic and hypoxic, this is a good place for a cell to die that is dependent on glycolysis for its energy, because acidosis and hypoxia will decrease the amount of glucose degradation. Howell-Jolly bodies are nuclear remnants.

Normally circulating Heinz bodies, and a massive increase in Heinz body formation under oxidant challenge, again are things that are similar to G-6-PD deficiency, but are obviously not 089 G-6-PD deficiency. They are general features of all the black rhinos that we looked at.

**Miller**: Just one side comment, in Christine *Hofney's* book she just made the observation without any description, that she had 092 that Heinz bodies were normal findings in white rhinos. The numbers were very small and she just left it at that.

**Paglia**: So she has done the work, I have not done this in white rhinos.

**Miller**: Yes, I do not know if that means anything because her numbers are very small, but to just throw it out.

**Paglia**: I am glad to hear that, because I suspected that to be the case. So at least now, after six years or so, we have enough baseline information on the species in general where we can start making some hypotheses and testing them. One of the obvious ones, was if these cells behave like G-6-PD deficiency, is there anything that we know about them that might cause that metabolically? Of course we have got this prominent finding of very very low ATP. So a natural question is, since ATP is required by hexokinase to phosphorylate glucose, does the relative deficiency of ATP produce a rate limiting reaction, not a G-6-PD deficiency, but one step before it? So that when there is an oxygen challenge and oxidation of the pool of glutathione, and a change in the ratio of the pyridine cofactor, which would normally stimulate increased glucose metabolism to the shunt; does that get blocked because there is not enough ATP to generate the substrate for that? That was our hypothesis about two years ago.

We tested that by looking at ways to change the ATP content of the rhino red cells. We did a whole series of incubations under different circumstances and with different substrates: 6 carbon sugars, 5 carbon sugars, all the 110-triases, to see what would happen. Could we change the adenine nucleotide content, principally ATP, by any incubation conditions with single or combinations of substrates? This shows that no additives produced... that the amount of ATP is stable, it starts at this very low value, remember humans are up around here, but it is stable over this incubation period. If we incubate the cells with glucose, we think well, they metabolize glucose, this might help. Nothing. They looked at it, they will eat it up, they will degrade it, but it does not change the ATP. This is also true of some 5 carbon and 4 carbon sugars. They can utilize them but not very rapidly, they are not very adamant about it. Not a lot of activity goes on in terms of glycolytic rate. When we start adding purine compounds though, this changes a little bit. Adenine is just a pure base of ATP by itself. It gives a little increase, which is normally not significant, but this is so consistent that I am sure it is. If we add the nucleoside with just the ribose attached to the adenine, we get a very perceptible increase of the total nucleotide. This is phosphorylating adenosine directly and forming AMP which is then getting re-phosphorylated up to ATP. If we add a carbon source in addition to the base, then that increases. Here is the curve for adenine plus glucose, which is more than just additive. This is producing a reaction that is quite perceptible. The best combination we have found is adenosine and glucose. Those two in combination allow over a period of time, the ATP to be built up to above human levels. So this has given us now a tool where we can start testing a few things.

One of the things I would love to test, we will get to a little bit later, that is will this change the infectivity of intraerythrocytic parasites, like Babesia? But we have to explain, or Raoul [du Toit] has to explain, why these rhinos have evolved this way. That is a very bizarre thing to do. The only other animal we know of is the echidna that is done that way. It is just on the border of mammals on that scale. We must have an evolutionary pressure. Again, if we use G-6-PD as the paradigm, as we will see later on, there are a number of reports that indicate that the reason that 200 million people around the world have maintained G-6-PD deficiency, is because it provides a selective protection against the most malignant form of *falciparum* malaria. So evolutionary pressure can look at the worldwide distribution of malaria and G-6-PD deficiency in the same geographic distribution. The evolutionary pressure there is a trade off, certainly an advantage in favor of not getting malaria; but you run the risk of having an acute hemolytic episode with fava beans or something else.

This is one way we can look at a similar hypothesis in rhinos, if there is a standard quantitative test that can be devised for infectivity of any of these, we can change the ATP content and see if that does effect it. We are now getting into the place where we can make hypotheses and test them. So this looks like two potential salvage pathways which we know are important in humans. At least this one is important in humans, this one is not. People who can not do this, take adenine to ATP, do not have any hematological problem that we can detect. People in whom adenosine is not available for one reason or another, do have a problem, so this is an important salvage pathway in humans. It is clear that the rhino relies on it to maintain that very precariously low amount of ATP that it does have. So anything that affects the enzyme that does this, is a potential problem for the rhinos. I am not going to show anymore data on this, because it is still shaky; but what we have seen is that the activity of this enzyme in the black rhino tends to fall into two groups, one with about ten units and one with about four or five units. That suggests heterozygosity of these *four* deficiencies state that might put them in particular jeopardy. That so far is about the only indication of a subset of black rhinos that might be in greater jeopardy of hemolysis than the entire species. At this stage, I have to go with the feeling that general species characteristics are what we are up against here, and all of the black rhinos are likely equally susceptible to these processes and conditions that produce hemolysis. Our original hope was to identify a single group with a specific enzyme deficiency, be able to measure a carrier state, keep those out of the breeding pool, and go on with the captive breeding. I do not think we are going to get that *for you folks*. This is a very unusual situation. But I think that is where we are.

**R. Kock**: There is some evidence for whites having lower AMP and ATP.

**Paglia**: Yes.

**R. Kock**: Is it just from the parasite point of view, that the whites obviously are not in a high *challenged* area generally for things like trypanosomes and stuff. There is some reason they are more susceptible. I do not think there is enough evidence 176.

**Paglia**: Yes, Raoul [du Toit] and I have talked about this in letters over the years, because some of the interesting differences that you have mentioned in terms of infectivity to parasites, and topography where they are going. I think what is particularly interesting will be the plants that are available in those areas which we will get to in a moment.

One of the ways we tested this early on, was to prime red cells as I showed you before, by *cooking* them with adenosine and glucose, building up their ATP's and then challenging them with an oxidant and seeing if that changed anything. We did three rhinos together, and this is a mean of their curve. When they are challenged with acetyl phenylhydrazine, their glutathione concentration fell down in just the fashion that a G-6-PD human would do. The normal human control should give you that kind of stability under these similar conditions. When we primed one of these rhinos with ATP, and tested it just at this point, we got that. We went, wow, that has got to be the answer. ATP is rate limiting. So we did it again with another one of those other rhinos that we still had blood on, and got this curve. It still looks like that is what is happening. That is the good news. The bad news is that we have since that time, not been able to repeat that with any other rhinos. No matter how much we prime them, they all seem to fall at that seem rate if you run them as paired animals together, so that you have an internal control and there is no question about running them against other animals as a mean. I have little separations, but now we are back to square one on that. I do not think that ATP concentration alone is the... It is not that simple. I do not think that is the rate limiting reaction. Again, I do not have an answer, only questions.

Let me just run rapidly through these [slides]. This was a series of cartoons and a story board that I did to try and put all of this together so that others could see conceptually what happens when the ordered structure of a membrane is attacked by oxidants. We have the situation where the "PAC-man" glutathione comes in here and instead of allowing these oxidants to destroy the components of the cell, it comes in and gobbles up those oxidants, in turn becoming reduced itself. That shows then that we have a finite amount of that particular antioxidant that can go out and do this. Being that it is a finite amount, we have to have a mechanism to regenerate it if the cell is to go on living under constant oxidant stress. This is just summarizing what we talked about chemically. So as those glutathione molecules, tripeptides, are oxidized gobbling up these, then there is a process in the cell where by these are taken back in and regenerated back to the reduced form. This pool is then allowed to stay constant. This of course requires energy, so all of this metabolic machinery that we have been talking about provides the power to produce that regeneration of the constant pool of glutathione. Of course the fuel that runs the engine is ATP biochemically. Here we see a full tank, which is characteristic of what humans have, and all other mammals. In the rhino situation however, this rapidly changes and we get this situation where we are just working with a very small amount of fuel; so that the reserve is dependent on this, running near empty. One way we can generate more ATP is through glycolysis, taking glucose and distilling it and running in more ATP That can keep this precariously low level in balance, and so oxidants can be met unless they get to be too much. One way we

know that we can stimulate this process and make more fuel in humans and other animals is by providing phosphate. Inorganic phosphate stimulates glycolysis. So taking this as a hypothesis and doing some *in vitro* experiments where we provided increasing amounts of phosphate, we have shown that we could indeed produce this very same effect. By stimulating glycolysis, we could increase the amount of ATP.

These are three genealogies of rhinos that Eric [Miller] had worked up in his original descriptions. I have added this. This is "Olive" who died on Saturday. "Olive" had never had a hemolytic episode and she had three offspring who had died with hemolytic episodes of isolated kinds and places. The fourth had leucoencephalomalacia as I recall, and the fifth who is alive and well in Europe somewhere. So "Olive" was one of the earliest ones we saw, dating back to 1984. She has been an obligate carrier if we were looking for an enzyme deficiency. She had never had a hemolytic episode herself until about a year ago last July. We will get into that momentarily. That kind of genealogy suggests hereditary groupings that again are similar, not *x linked* necessarily, but similar to G-6-PD deficiency.

This just shows the world wide distribution of G-6-PD deficiency [in humans]. A number of papers indicate that a low ATP in those cells protects against malaria, or the difference in the nucleotide protects against malaria, a justification. We also of course have the body of literature that Eric [Miller] alluded to that indicates that horses as related animals, and other animals as well, are also subject to similar hemolytic episodes, and Heinz body formation, in response to eating certain foodstuffs. I think this is very pertinent when we discuss about what might be happening in the wild, particularly the point that Raoul [du Toit] and I have discussed a long time about, these massive die-offs. Is it possible? A good theory was given yesterday as one explanation, and these are all theories. But I just raise the question, is it possible that a drought had occurred at that time, that the only plants remaining to browse on were a selected species that normally might not be eaten in abundance. Or was it after the drought ended, and the first blooms happened to be plants that had compounds that contain a lot of these lysines, dilysines, or some of the other polycyclic hydrocarbons, or alkaloids, or aflotoxins; things that are normally detoxified by the glutathionase transferase and metabolism through that pathway. Is it possible that those provided a dominant part of the feed for a short period of time that would then allow a hemolytic episode to affect a large number of animals simultaneously?

**Munson**: As I recall the maple leaf, issue too is one of wilted maple leaves or cut maple leaves, not fresh ones. That might be related to how we feed too in captivity, that the browse hangs around and is wilted. So something not normally toxic might become toxic.

**Paglia**: That is absolutely right. So again, the similarities. It was on this basis that I originally sent out this notification to all of you and other zoos who had black rhinos, suggesting that even though we had no basis for it other than this almost anecdotal information, that these animals ought to be treated as though they are equivalent to humans with G-6-PD deficiency, and that certain things ought to be avoided; obviously infection, conditions that suppress glycolysis such as acidosis, such as hypophosphatemia. All of

these ought be judiciously corrected clinically. Certainly all of the compounds that are known to produce hemolysis in humans with G-6-PD deficiency, ought to be avoided or used only in the most rigid circumstances. In trying to think of a number of possibilities, things like Naphthalene which is used in a lot of cleaning solutions and soaps, other phenolics that occurred in here that could be in wood preservatives, insecticides or organic cleaning solutions, or rodent control things like they put around zoo pens. We tried to think of all of those possibilities. That was why we mentioned wood preservatives in creosote. We were not thinking about creosote toxicities specifically. We were looking at warning against it only in terms of its relation to other cyclic hydrocarbon compounds that were known to produce hemolysis in humans.

These were some of the papers relating to phosphate. In humans, hypophosphatemia is associated with a fall in red cell ATP, a fall in red cell glutathione, and it can produce hemolysis. Conversely, in humans with increased phosphate, their red cell ATP goes up. Since we had been playing with this *in vitro*, we got to wondering if this were indeed due to the low levels of ATP, could we provide assistance to a rhino in distress by making it hyperphosphatemic and increasing its red cell ATP? That was the hypothesis, and we got the opportunity to test that on "Olive" a year ago. We actually had the opportunity with Evan [Blumer] and Janet [Stover] and some of the Zimbabwe animals that came in earlier that spring who had gotten into trouble. We tried tentatively to help them out with phosphate therapy. But we did not have an opportunity, because they were in jeopardy, to do it with enough frequency that it would make a real difference. They could not be phosphate loaded fast enough for us to be able to detect any change in their red cell ATP. But the idea was there, and there was a sound laboratory basis for attempting it as a fundamentally sound reasoning, at least we thought so. Then in July, "Olive" had her first hemolytic episode. She was in her 40's or so. She was also having problems I think at that time with ulcers. At Oklahoma City Zoo they were amenable to trying something that might be risky, but they thought it might be worth it. We all put our heads together and decided to try intensive phosphate therapy. We did not have anything else to offer.

This shows the time course of the events that happened. This is showing the total of ATP, ADP and AMP. The shaded area is the normal range for black rhinos. We did not assay her actually until 1990, but we had seen her earlier on in 1984 and 1989. Her hematocrits were up in the normal range for rhinos at that time, and her serum phosphate levels were in the normal range. When the crisis occurred, the first sample showed that she was extremely hypophosphatemic. She dropped down to 0.6 or 0.7, I think. Her hematocrit had fallen precipitously down. This is where we came in and with the first samples, measured the ATP *and they all counted* at the lower range. But then they started daily or every other day, to knock her down and intensively infuse phosphates. Each of those arrows is one of those parenteral infusions. It did not immediately correct her serum phosphate, but her hematocrits stabilized. She stopped hemolyzing. We were able to detect a spike with three values determined here in her ATP range, that took her up almost to human levels for a short period of time before falling back into the normal range. She started looking better and stabilized and got clinically better and her hematocrit started to come back up. So they stopped

the parenteral and then picked up on an oral supplement of phosphate that was given at this time. She returned her serum phosphate levels back in and her hematocrit back to normal range. It looked like we had had a reversal, a cessation, an interruption and a reversal of the hemolytic episode. What animal? Would it have happened to anyone? I do not know. Again, questions, not answers. Following along though, we have been able to measure the ATP levels, and they are in the normal range or above with this phosphate therapy. So this was a good indication that we had done something.

Then recently her feet started getting worse, the ulcers, she was bleeding, she was losing blood. She started going down hill, her hematocrit fell rapidly at that stage, and the phosphates again came back down. She was still measuring high ATP. This is where she died last Saturday. The last two samples arrived the day before I left to come here, so I do not know what this [ATP] would do. Again, I can not say this is a cause and effect thing, but it is promising. Joe [Smith] mentioned Ernie *Boyd*. Ernie is sort of one of the grand ol' men in human red cell metabolism who did a lot of the G-6-PD work; and he had a say in these anecdotal case reports that one rabbit is no rabbit, but if a bear says a single word, it is a talking bear. In our lab we kind of refer to talking bear experiments or talking bear situations. I want to hear this word so badly, that I am hearing it but...

"Marsha" at the Dallas Zoo has been on oral supplements of phosphates and we have been able to follow her. Again, she was an animal with severe ulcer disease that was eventually euthanized, but we were able to follow her. She was anemic, but not hemolyzing. Here is her hematocrit in the 25 range coming through here, hemoglobins less than 10, phosphates that were low. This is the amount of daily oral phosphate supplement, I think this is the equine preparation that you want to use, *Hy-Phos*, something like that. She was getting large amounts here when her phosphate came back up. We were measuring ATP levels on periodic samples. This fuzzy area is the normal range, and she was up. They dropped the dose here, thinking that she was getting too high, and she immediately fell down and we detected a bit of that too. They increased it to 90 gm daily, it came back up. All the way until she was euthanized, she had shown a significant increase in the amount of total adenine nucleotides in her red cells. So I feel very confident that this was doing something. Now whether it had anything to do with her anemia or whether it was going to protect her from the hemolytic episodes, I do not know. Interestingly, the serum phosphates do not necessarily correlate with this, and I have to raise the question about how accurate the serum phosphate level really indicates the available phosphate pool.

I have to address the issue of catalase, because I think this is one place where we have found something that is different enough between white rhinos and black rhinos, that it may be of some significance. Catalase is the most active enzyme present in human red cells. The human values are up close to the 50,000 units, an enormous amount of activity. These are three standard deviations below the value. This of course is the reaction *of catalase*. When we measured black rhinos, this is the mean value we got, just about 2,000 units. We have recently been able to get assays over the period of the last year and a half on five white rhinos. They are in a range that is very significantly higher than this, it is sort of intermediate

between humans and the black rhinos. This is the range that we see heterozygous deficiencies of given enzymes in humans. People who operate with 30% or 50% of the usual amount of a given enzyme do *not* have a clinical problem. You have to say how much of an enzyme do you need to define it in those terms? We do not know that, but it looks like there is a significant difference in the catalase activity between the white rhinos and the black rhinos. The whites are intermediate between the blacks and the human.

Interestingly, when I was putting these data together, I went back through and looked at the individual values. The ten rhinos that came in from Zimbabwe last year, had the ten lowest values with a mean of less than 900; compared to all of the other black rhinos exclusive of those which had a mean of around 2,000. Is that significant? It makes me wonder, I think conceivably it is. Then in looking through the individual values I noticed that "*Quanza*" who was presented to you yesterday by Dave Kenny with leucoencephalomalacia, had the lowest individual values of any of the animals that we have measured catalase in. She also had the lowest glutathione peroxidase levels. As I mentioned during Dave's [Kenny] presentation, there are some human neurologic degenerative conditions in which glutathione peroxidase has been *incriminated.* So again, we have some circumstantial things that have occurred that make me wonder, does catalase deficiency have any basis for the problems that we saw in "*Quanza*" or the problems that have seen in the recent shipment of Zimbabwe black rhinos.

Human catalase deficiency was discovered by Takahara who was a head and neck surgeon and had a patient come in ***and child*** with gangrenous ulcerations and erosions of the gingiva, mandible and maxilla. He debrided this wound and was expecting to flush it with hydrogen peroxide. When he did, it did not bubble and it turned black. He thought, oh my God, someone gave me silver nitrate instead. So he got a fresh bottle of hydrogen peroxide and did the same thing, irrigated the wound with it, and again it did not bubble and it turned black. So he put two and two together and got 17, and discovered human catalase deficiency. The dominant form occurs in the Japanese. These are drawings of mandible and maxillary teeth showing massive erosions that were really truly gangrenous ulcerations. Clearly not what Linda [Munson] was presenting to us yesterday. Gangrenous ulcerations and necroses that eroded through bone into sinuses, etc. Lesser involvement of course was also seen. And as more and more attention came to be directed towards these cases, prophylactic oral hygiene and all helped, and the incidence of these ulcerations was reduced to almost nil. So what was probably occurring was the initiation of pyogenic bacterial infections that were not being neutralized, that were generating peroxides and not being neutralized by catalase which was deficient in the tissues, and allowing a normal progression of the pyogenic reaction to occur. That is clearly not what is happening. But of course I did not know what Linda [Munson] had been doing. When I had found the catalase deficiency, I remember Dr. Takahara 465 symposium presenting that and immediately felt that there had to be a connection. The chance of this occurring fortuitously seemed too remote.

I had the opportunity to actually see my first case with Evan [Blumer] when he cordially invited me to help him give this necropsy on one of the Zimbabwe animals. This is the liver coming out under the

472 margin. This is hemorrhagic skeletal muscle. This was my first look at a rhino necropsy and clearly this was not a hemolytic death. This was a primary *lymphatic* problem at least, with evidence of hemorrhage everywhere. We immediately had to go with the assumption that if hemolysis was occurring it was a secondary phenomenon. Just looking at that liver made it seem like the most likely source. The sequence of events resulting in reduced body factors, you end up with a hemorrhagic terminal event. That is the way I as a pathologist would have put it together on just gross examinations. I had not seen any of the micro at that time. This was the liver showing that black green discoloration. I was very curious to see if I could repeat Dr. Takahara's findings, and so we took a little bit of hydrogen peroxide and put it on various tissues and it bubbled. I thought, that is interesting. I tried it other tissues, this is some of the hemorrhagic muscle, all of that white area is throbbing. Serosal surfaces, the epithelium, the vessel, I tried those as well. Every thing seemed to throb, not rapidly, not immediately; but enough so that I thought there has got to be catalase activity here. The other tissues do not share the red cell defect. Except when I finally got back home I thought, well I have not done that with red cells, and is that small amount of residual activity enough to produce the bubbling? Indeed I tried it on red cells, and it surely throbbed. You can do a quantitative assay and they are very efficient. So we have not looked at catalase activity in the tissues and that is an extremely important thing that we have to do--try to set up the assays and adapt a partner with others who have had more experience with tissue assays other than red cells, and that is for the future as well. So there are a lot of things left to be done.

The white rhino I think provides some final and if I may say some interesting information. This is going back to that slide we started off with, if everybody were the same as humans, there would be a straight line across here. These little bars indicate where we have now looked at between five and ten rhinos, this is a mean of ten for most of these enzymes, to see how they compare in this same enzyme activity relative to humans and relative to the blacks. You see on those that go up for the blacks, the whites also go up, perhaps not quite as far. But the hexokinase, G-6-PD, and glutathione peroxidase are all elevated; and whereas those that go down in the blacks, also go down and a little bit further in most instances for the white rhino. The one exception is catalase where the white rhino is significantly higher than the activity in the black rhino. Again, I do not think there accurately detectable activity of glutathionase transferase in either the white or the black, in fact if anything it is lower in the white than in the black.

This last slide just puts it all together. This is that same shunt going through G-6-PD, the cycling glutathione and pyridine cofactor. This is where glutathione peroxidase and the catalase and the dismutase that we talked about yesterday all come in. If we are looking for a common area of focus, we have all been looking at various aspects of it, I think it is this metabolic pathway right here. I will point out again that the glutathionese transferase is the one that normally, using glutathione as a cofactor, detoxifies a lot of what 547 calls the *zeno*biotics of nutrient metabolism. Plant metabolites that potentially would be oxidant formed are neutralized and cleared by glutathione-S-transferase. This is virtually absent.

So that is step one. I think again our area of concentration ought to be that pentose phosphate pathway and antioxidant metabolism through whatever means that we might be approaching it from different angles. This is for Carol [Bolin], we were discussing some of this last night and I mentioned that one of the great joys I have had in my professional life has been the ability to move from a field of traditional medicine, with constraints that I am sure you have all experienced from the point of view of being a patient yourself or associated with other physicians, but things that have moved me to wonder how quickly I could get out of medicine. The experience of working with you all and what you have been doing in a project of this magnitude has been one of the more rejuvenating experiences in my life. So I am grateful for the opportunity to be here and be a part of this. Thank you very much.

**END**