VITAMIN E, BETA-CAROTENE, ESSENTIAL FATTY ACIDS AND OTHER BIO-PROTECTANTS

Rob Stuart

TAPE 6B 129

Stuart: I make my living basically by detailing bovine and equine veterinarians. I feel comfortable talking to those people. I feel very uncomfortable talking to you, because I feel like I am the duck out of water. I will try to walk through this. My background is basically like Bill's [Sadler], ruminant nutrition. I have been working in the vitamin area since the early 80's. I was involved in some early beta-carotene work for the lactating dairy cattle. I have presented papers on that at dairy science meetings. Our biggest hurdle to overcome was cost, so basically the project was dropped because the dairymen could not afford 30 cents per cow per day to supplement beta-carotene. So this has been an area that I have been interested in for the last ten or so years, but because of cost for the food producing animals, beta-carotene addition to diets has basically been dropped. I think it is an area you need to look at. I am going to make very few recommendations. I am going to make a lot of comments in regards to questions and let you decide in which direction you should go. I know Ellen [Dierenfeld] and I have been visiting. I first meet her in 1987 at an Italian restaurant in New Jersey. I was working for 149 Roche at that time, and Ellen [Dierenfeld] had been doing a lot of cooperative work with Roche. In the last couple of years I have been suggesting real strongly that we need to look at beta-carotene status in captive animals compared to just straight retinol status.

So with that, I will begin with a brief introduction of the lipid oxidation cycle that can occur. Basically the first step is the formation of a free radical. That occurs constantly in all cells essentially. The factor that we always need to consider is that the addition of oxygen creates that potential problem with the 156 polyunsaturated fatty acid. That in itself is not a major problem. The problem occurs when this chain reaction starts. It is kind of like throwing a ping pong ball into a group of traps, and they just start rapidly creating a sudden decline in cell wall integrity. If you continue on, the damaging reaction occurs when that free radical reacts with the lipids, protein, nucleic acids, and the result is you do have damage to the cell. The function of vitamin E is to basically cut the free radical off at the pass and create the ROOH again. The created product here is nonreactive. The subsequent vitamin E molecule that is utilized can be regenerated by the addition of vitamin C 168. The only problem about the regeneration point is there is a speculation that that may be more of an *in vitro* procedure than an *in vivo* situation. This is the type of information that is presented. If you have been in the human area or have read the popular press, there is a tremendous amount of interest in the antioxidant vitamins E, C, and beta-carotene as to how they relate to tissue health and other potential diseases in humans.

Look at the several protectors within the cell. There are several enzymes as you mentioned before. I am going to spend most of my time on vitamin E and beta-carotene, and essentially no time on vitamin C, because it is still an unknown as far as I am concerned as determined requirements for that vitamin. But, it does not mean it should not be looked at in various experiments. Looking at the three antioxidant vitamins that we mentioned... Vitamin E is a fat soluble vitamin. It is membrane bound, in that it seems to be associated with the lipid bilayer structure in all cells. It mainly acts as an antioxidant within the cell membrane. Vitamin C is more soluble and seems to be floating around in the cytoplasm, and mainly functions in and around other water soluble antioxidants. Beta-carotene, it depends on who you talk to, some people say it is not a true antioxidant; it is more of an oxygen quencher, for it prevents *single* oxygen from being formed. It in itself is fat soluble and the researchers are not exactly sure where it is occuring in the cells. So the exact mechanism of beta-carotene is uncertain.

Moving to vitamin E. There are basically two structures of vitamin E that you will normally see. The first is alpha-tocopherol, which is the biologically active form of vitamin E. The whole structure of the molecule *is beside* this OH group here, in which it allows the giving up of that hydroxyl group to the free radical. The 195 side chain allows the molecule to nestle within the lipid bilayer structure, and in so doing it *is bound in the membrane*. The commercially available form of vitamin E that is normally sold is alpha-tocopherol acetate. What happens is through an acetalation process, the acetate group is added and when it is subsequently consumed by the animal, the acetate is removed, and the release of free tocopherol which is subsequently absorbed. Taking the available forms of vitamin E one step further, there are two major sorts of synthetic vitamin E, which is made up of three different sources 205 alpha tocopherol, 206 alpha acetate, which is the form that is normally used in feed supplements and complete feeds, 208 tocopherol succinate is a powder and it used in baby formulas and products like that. The natural source vitamin E, there are three again available there. The natural alpha-tocopherol is the *highest* biological activity per milligram of all the products. This is a formula we use in a lipid vitamin E product that is commercially called *M-cell*. I will spend a little bit of time on that product showing you some of the results we found there. We also have a feed product called alpha-tocopherol acetate, which Purina Mills uses in a lot of there products as a natural source of vitamin E activity. The succinate again is used in various milk replacers for human babies.

You will have to bear with me on some of the data that I have, because most of my emphasis has been on beef and dairy animals. I am showing you the types of numbers we get in ruminants. *Sam Oregon,* at Oklahoma State, went out and bleed some beef cows and she also bled some dairy cows at the same time that were in confinement. You can see the values we got, 0.88 mg/dl which corresponds to approximately 8.8 μg/ml. The range was 7 to 11, and do you remember what the numbers were in the rhino? Below 1.0. So we are seeing a tenfold difference in circulating levels in beef cows compared to your rhinos. In the dairy cow in confinement, you can see the three or four fold reduction in vitamin E status. That is one thing we see routinely. If you take an animal off of grass and put them in confinement, their

vitamin E status goes down dramatically. It goes down rather quickly. Looking at another group of animals that we routinely work with, that is calves at arrival at the feedlot... If you talk to most veterinarians they offer the same program of fortification, irregardless if they are beef calves or Holstein calves or what we call the Holstein steer calves that come off the calf ranches. Looking at the serum tocopherol level in the feed calf, it is 6.0 μg/ml versus less than 1.0 for a Holstein calf. If you look at the husbandry practice for the Holstein calf, basically they never see grass. They are bought at dairy auctions at one day to a week of age. They are sent to calf ranches and never see grass. If you look at the serum retinol values on the feed calves versus the Holstein calves, you can see that the beef calf has a value of approximately 17.0 compared to 7.0 to 10.0 for the rhino, and the Holstein calf is up to 42. I was amazed at the high levels in the Holstein calf, until you look at the levels of vitamin A that are feed to a Holstein calf that is in a calf ranch--very very high levels. These are dated from El Centro, we were talking about El Centro, California and Richard *Zen* is in prison down in El Centro. Another thing we learned about vitamin E status in the dairy cow, is that in certain points in the reproduction cycle, vitamin E status does go down dramatically. In this case, this is Ohio State data showing that at calving in the dairy cow, vitamin E status does go down. There is no explanation for this What we are attempting to do now is during that time of parturition we are trying to bridge that valley that occurs in vitamin E status. So I do not know what happens to your female rhinos when they give birth. Do they have that drop in vitamin E status? The same thing occurs in swine as well.

Dierenfeld: What is happening then?

Stuart: I do not know, I do not know why it happens.

Dierenfeld: I thought it was going into milk to go to the baby.

Stuart: The quantities there would not represent that. That does happen, it is a good point Ellen. In swine and cattle, colostrum contains much higher levels of vitamin E and other fat soluble vitamins compared to regular milk. Does your rhino in the wild ever produce colostum or does she wean her calf before she gives birth or not?

M. Kock: No, when she gives birth, the other calf will still be with her, but the calf is a subadult and should be weaned probably at two or two and a half years.

Stuart: So they do have an opportunity to produce colostrum.

Kenny: Have they ever measured [vitamin] E in calves?

Stuart: Yes, that has been done. Usually in ruminants and in swine the [vitamin] E level at birth is very very low because of poor placental transfer. The colostrum provides a mechanism to build vitamin E status in the neonate, provided the dam has received adequate vitamin E through gestation. If she does not get vitamin E, then she is going to be low at calving, and also the calf is going to remain low because there it is not any going through the colostrum. But, I will not say that that is due strictly to the build up in the colostrum. I think it is deeper than that. I think there is other factors that are playing a role in that situation.

We are looking at the ability of animals to use natural versus synthetic vitamin E. This work was just published this summer at the Animal Science meetings. This work was done by the University of Idaho. What they did basically was to supplement three sources of vitamin E; either synthetic vitamin E which was the 282 tocopherol acetate, which is normally supplemented; we compared the *M-cell* product to that which is the 4,000 IU of natural; and also we injected 4,000 IU of natural tocopherol. You can see the peak milk values were dramatically improved with the injection, which is what we normally see because that is the most efficient way to rapidly improve vitamin E status. The thing that surprised us was the 21 day milk tocopherol secretion values, 13.0 mg above the control in synthetic was fed in one dose. When we supplemented the 4,000 IU of natural tocopherol we got a value of 27.0 mg. I did not put any significant values, but this was significantly better or higher. The natural injections produced a value of 110.0 mg. So you can see that there was a difference in how the animal was able to metabolize the natural source compared to the synthetic. This is why we are going around trying to convince companies to switch from synthetic vitamin E to natural in these critical type diets. I think Purina Mills has made major steps in that area. They are one of our primary customers in the use of natural vitamin E in their products.

Early on, I visited with Ellen [Dierenfeld], and she made us aware of the Phoenix Zoo that had some elephants that they were bleeding weekly. We put the *M-cell* into those animals at 2.5 IU/kg of body weight, which is what she was recommending; and looked at the repletion/depletion type program where we put it in, took it out, put it back in, into these three female elephants.

Blyde: This is just oral supplementation?

Stuart: Yes, oral. And you can see that the initial values were approximately 0.2μg/ml. Within a couple of weeks we showed a dramatic improvement. This is one of the things that we have noticed in several species that we have tried, they are able to utilize this *free* tocopherol form of vitamin E. As you take it out you can see the drop, and then putting it back in it does go back up. We have two rhinos, these are white rhinos at the 316 Park Zoo in Tucson. I should point out that Ellen's [Dierenfeld] lab developed the analysis for these samples. In this case they were supplemented with approximately 6 IU/kg of body weight. You can see that they rose from approximately 0.35 to approximately 0.8. Then the product was removed at this point. This was the female. The next sample was approximately a month later. It remained higher, then dropped down the next month's bleeding. The male white rhino did not continue out as long as the female did, I have no explanation for that. But you can see that we did show good improvement in vitamin E status when they were supplemented with the *M-cell* at 6 IU/kg body weight. You should not be concerned about tolerance levels. Vitamin E is not like other...

Miller: The body weights on those rhinos, where those accurate weights or estimates?

Stuart: I am not certain.

Miller: I might suggest when you are doing this per kilo that you check. We thought ours were 3,000, but when we put them on a truck scale, we have a average size male that was 2200 lbs. So it would be almost 50% difference. It surprised me.

Stuart: That is a good point I will check and see exactly what those weights were. It is interesting, they were able to bleed these without restraining them. They were able to go in and hit the blood through an ear vein without restraint. I have a neat video tape on that. One thing we need to be concerned about is the toxicity of vitamin E. Because of its role in the animal as an antioxidant, the animal is able to tolerate high levels. What we see in pasture situations, is we have seen levels in cattle as high as 17 or 18 and as low as 0.2. So we see a tremendous variability in vitamin E status, and basically within one quantity consumed. So I would say that vitamin has a real high tolerance level and you should not really be concerned about toxicity in this vitamin.

Let us move to an area that is a little red from the perspective of what it is. Beta-carotene is a natural pigment in various vegetables and fruits and I should also add grasses. This was put together mainly for humans. It is partially converted to vitamin A in the body and the rest is stored and utilized as beta-carotene. As Ellen [Dierenfeld] mentioned earlier, an animal out browsing or grazing consumes zero vitamin A activity. It is all beta-carotene and the animal converts the beta-carotene to vitamin A activity. In the rat, 1.0 mg beta-carotene is equivalent to 1667 IU, in the ruminant we use a value of 400. It is kind of a guesstimate, we do not really know. In swine it is approximately 800 or 900, and I think about the same for the horse. We do not know what the conversion of beta-carotene to vitamin A activity is, but we do know that animals out on grass never suffer from vitamin A deficiency. They are always have adequate vitamin A status.

If you look at the structure of beta-carotene, it is basically two vitamin A molecules put together end to end. Vitamin A is typically supplemented as vitamin A acetate, again this is a stabilized form of retinol. Beta carotene can be available in either its natural source which is *cis-trans* beta carotene or synthetic beta-carotene which is essentially all *trans* beta-carotene. The major difference between the two sources is solubility and the percent of absorption. The natural seems to be absorbed much [more] readily because of the mixture of *cis* and *trans* and its solubility is much greater.

Looking at some absorption patterns in humans, we can look at the fact that approximately 12% of the dietary beta-carotene is absorbed and is taken into the lumen of the intestine at the wall level. Part of the beta-carotene is converted to vitamin A ester, primarily vitamin A palmitate and then taken to the blood, to the liver and stored in that fashion. The remainder of the beta-carotene, as we mentioned earlier, they are not certain exactly where it functions in the cell. One thing you can do if you have ever taken blood from animals out on pasture, what is the color of the serum? A real golden color. That is primarily due to carotenoids that that animal has consumed.

The important biological effect of beta-carotene is the fact that it does alter vitamin A activity and the fact that it is the major way for the cells to *quench single* oxygens which are the beginning steps of some of these reactions that occur in cells, and when the free radicals are generated. That activity, besides its pro-vitamin A activity, I think is very very important in the animals that you are working with. Here is some data on what we see in animals. Just ignore the treatments, but from the standpoint that we were looking at various injectable formulations. As cattle come in from the feedlot, and these were approximately 450 lb beef calves, the value was 50μg/dl. You can see within seven days it dropped down on an average below 30. So the disappearance of beta-carotene is very very quick when you take an animal off of grass, put them into a feedlot situation and feed them stored roughages. I think that is one thing you need to remember too. If you take your rhinos off of browse and put them on a prepared feed, I think their beta-carotene status will drop very very quickly. I do not know that, but Ellen [Dierenfeld] is going to find out.

Dierenfeld: You lose about 90% of your beta-carotene just in the drying process also, the same as vitamin E.

Stuart: Yes, that has been shown in hays and silage. You do lose essentially all of your beta-carotene activity.

Blyde: Is it true that if your hay is still green, is there still beta-carotene in it?

Stuart: Yes.

Dierenfeld: Is it as active as fresh? No. You can not associate 415.

Stuart: Hays basically continually decline over time. Whereas silages seem to decline rather quickly and then once the silage is sealed.these have a very level effect when you sample silage versus hay. But hay is just a steady decline in beta-carotene.

Blyde: How long does the beta-carotene last in cubes?

Stuart: Basically everyone uses a value of zero when they are supplementing animals with vitamin A. They are saying that feed will basically deliver no vitamin A activity. So they basically discount it totally. There is some there, but the variability is tremendous. We have done a lot of analysis of samples, and again the variation is very very high. So usually the nutritionists will give a value of zero. Is that right?

Sadler: Yes, when you go through a pellet mill which is steam heated, compressed, and then through a dryer or cooler situation, you have got to basically say you just do not have very much of it left.

Blyde: So if you just feed cubes and hay...

Sadler: Most commercial feeds are supplemented with vitamin A acetate added in the diet. So there is vitamin A activity in the product, there is not much beta-carotene. You have to separate the two.

Stuart: Showing the potential differences that you can show in the beta-carotene and total carotenoid status in animals... This work was done at the University of Florida. This is a Master's thesis, 440 work. You can see that in group #1 those animals went directly to the feed lot and were on feed for

138 days. Look at the mean liver carotenoid level, 1.80, the mean liver beta-carotene level was 0.79. Group #2 spent no days in the feed lot. Look at the total carotenoid level, 13.0 versus 1.8; beta-carotene status in the liver is 7.7 versus 0.79. So you can see a ten fold difference in the beta-carotene status in the livers of those animals that were maintained confined, compared with those that were out on grass. Groups #3, #4 and #5 were maintained in feed lot situations for various amounts of days. You can see the longer you take an animal away from grass, their beta-carotene status does reduce.

One other area we have been involved in is the effect of goosypol on serum beta-carotene, alpha tocopherol, and retinol status in cows. We also have the data in their calves. This work was done at Texas A&M, Ron *Randall's* group at Overton. They basically were looking for reproductive failure in the cow when you feed her gossypol. In general they show no effect. Basically the cow was able to tolerate the consumption of 4.0 gms of gossypol per head per day. The interesting thing is when you look at the impact of goosypol on alpha tocopherol status and beta-carotene status, and if you look at the retinol values... These cows again were out on grass, this would have been Bermuda type grass and this would have been spring through September, they were fall born calves. The control cows getting no gossypol had a value of 2.59 mg/dl or 26μg/ml of tocopherol, so very very high levels. When you fed the cottonseed meal there was a reduction. It was not significantly reduced, but you can see a trend toward reduction. Retinol was not affected. Beta-carotene was essentially cut in half, and that was significantly reduced. As you can see as a calf *and pass through* calving and 20 days post-calving, beta-carotene status did drop down to approximately 700μg/dl. So there was some impact, but it did not seem to be as dramatic in the cow as it was in the calf. That calf was nursing the cow that was being fed the cottonseed meal. These values are for the calves. They did not get any goosypol, they basically were suckling calves. They only source of vitamins that they received was through the milk. At calving, retinol was not affected, we looked at 0, 7 and 28 days. Alpha-tocopherol as you can see was affected, it went down from 2.3 with no gossypol; and when we fed 4.0 gms to the cow it went down to 0.102. The beta-carotene status showed dramatic reduction. Now, what this means we are really not sure, but it gives an indication that when you feed phenolic compounds to the cow it does impact their vitamin E and beta-carotene status that is being transferred through the milk.

Safety features of beta-carotene... It does not cause hypervitaminosis A, because its conversion to vitamin A in the body is limited. Only approximately 50% to 60% is converted. And the more beta-carotene that is consumed, the greater the amount that is excreted in the feces, so absorption is limited. In humans, most of the work has been done in that area. There have been some studies in which they fed beta-carotene up to two to three years and showed no impact on human health in relationship to supplemented 512 of beta-carotene.

Moving now to an area that I have more questions that answers in relationship to the needs of the rhino, because of their types of diets, is the need for polyunsaturated fatty acids. I should mention that if you look at the fatty acid profile of various vegetable oils, there is one that shows through to have high

levels of linolinec acid, and that is flax oil. There are others mentioned. There are levels from 0 to as high as 57% linolenic acid. I was talking to several people about that, and I think that there is a potential to look at that in the rhino because of the fact that you are again feeding processed fed stuffs to these captive animals; and the needs for 529 as far as for cell wall integrity and some of those things, are quite high. But you can not just say we are going to feed 532 with out increasing the amount of fat soluble vitamins, like vitamin E and beta-carotene. So the needs for those vitamins has been shown to go up as you increase the amount of 536 intake, primarily in pets. So the area of fatty acid supplementation through these various vegetable oils I think is one area that needs to be looked at. Flax seed oil is being tallied in the human area. Some of the problems that I think that I would be faced with would be cost and from the standpoint of whether it would show efficacy in the rhino, I have no idea. But as Bill [Sadler] is saying, you are using crude soybean oil, which is better than refined, because it has a lot of vitamin E compounds then and very little of them have been lost in processing.

Some of the other areas that I think we need to consider... Along the trace mineral area, I just put these together and this is one I think you need to look at, copper. But do not be too liberal with your copper supplementation, because that is a transition metal and it can create potential problems. Zinc I think is one that would be of interest to me, because of the fact that zinc is used in fatty acid synthesis to produce long chained fatty acids in the animals; so I think that is one that needs to be looked at. Cobalt is a question mark. I do not know if they need cobalt or if they need B12. Which do they need? They may not need both. I think excessive potassium is something that the ruminant people ignore most of the time. I saw that your ratio of potassium to sodium was 4:1. It is not uncommon to see that as high as 20 or 30:1 in certain feed lot rations. There might be high levels of potassium, especially in *starter* feeds. So keep your potassium low and your sodium as high as you can go. Magnesium I think is one that is of interest because I think if you get your potassium and sodium balance too far out of whack, then you have magnesium deficiencies. What is typical of cows on fescue, is grass tetany. It is primarily caused by a potassium and sodium imbalance. So what they do is supplement 575 in that situation. The question about trace minerals, to chelate or not to chelate, which should we do? The only one that I think really needs to be considered would be zinc. Zinc methionine has received a lot of research in ruminants and monogastrics and the data look very interesting. I think that is one that needs to be potentially considered, zinc methionine. You can get chelated copper, chelated zinc, you can get chelated cobalt. Those would be the ones, if you wanted to spend a lot of money you could chelate them all.

Dierenfeld: From profiles of amino acids in plasma of black rhinos, methionine is the first most limiting amino acid that has been identified by *Clinton Roger's* lab out of UC Davis.

Stuart: The level of methionine that you should get with zinc is usually so low, that it really would not have any nutritional impact. But that is one that I would highly recommend to be the first to be considered. B vitamins, thiamine, biotin, B12, and folic acid are the ones that I think would be the most

critical. I have not looked at the profile of yeast to see how much of those that they would add. Biotin is the one that is involved in skin, and zinc also is another one that is actively involved in making the skin 606.

Fat soluble vitamins... I think we should stay away from vitamin A completely. I do sell beta-carotene. We do not sell it presently, we are doing research on beta-carotene, primarily in horses and in lactating dairy cows. The approach we are taking is that before we ask a dairyman to invest in beta-carotene, we actually take a composite blood sample from the lactating herd and the general beta-carotene status, and from that draw conclusions to whether or not they should invest in beta-carotene supplementations. With your animals I think there should be no question, I think you should go to this. I really feel confident in making that recommendation. From the standpoint of the approach that I think we need to take, and what the approach is that we take, is that most of the nutrients that we supplement are so volatile, that we have taken the approach of supplementing the diet with vitamin E, for instance *free* tocopherol versus tocopherol acetate. This is the form that we recommend, it is the form that we developed in an injectable formulation. I heard mention of injectable formulations used. We had one that we launched in 1989 and last year Schering-Plough licensed that from Stuart Products and they are now marketing it in the Vital E line of injectable vitamin E products. So from the stand point of our approach, we are developing supplements. I think Bill's [Sadler] approach is that he is developing complete feeds. One of the things that you might want to consider, especially with beta-carotene, is if you put beta-carotene in a complete feed the percent absorption may be very very low, whereas if you put it in a *misolized* formulation, like our *M-Cell* formulation, your absorption may be dramatically improved. So just because you put a vitamin in the diet, it does not mean the animal is being able to utilize it.

Another approach that we have found is that neonates can not utilize the esters very effectively like vitamin A acetate or tocopherol acetate, they need the *free* alcohols rather than the esters.

Dierenfeld: Could you make a short comment on any animal studies of immune status and beta carotene?

Stuart: Beta-carotene and immunocompetency... I would say most of the work has been done in rodents. There was one study done in Kansas, Jim *Morell* and his graduate student. They looked at beta-carotene and they did not seem to show any dramatic improvements. I do not know exactly what parameters they looked at, but I remember basically no effect. They looked at all three, [vitamin] E, [vitamin] C, and beta-carotene.

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But the main function I think of beta-carotene in the rhino would be as an oxygen quencher, or something that would basically protect the cell from whatever oxidant is in that cell.

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