UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

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OPP TECHNICAL TRAINING COMMITTEE

OPP SCIENTIFIC AND TECHNICAL TRAINING COMMITTEE

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NEUROTOXICITY OF CHEMICAL MIXTURES:

AGGREGATE AND CUMULATIVE EXPOSURE

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SYMPOSIUM

THURSDAY
APRIL 27, 2000

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The Symposium was held in the Marriott Forum at 1999 Jefferson Davis Highway, Arlington, Virginia, at 9:00 a.m., Dr. Deborah Norris, U.S. Environmental Protection Agency, presiding.

PRESENT:

DEBORAH NORRIS
STEVEN GALSON
MOHAMED ABOU-DONIA
WILL BOYES
GREGORY CHRISTOPH
RICHARD HERTZBERG
HERMAN KOETER
VIRGINIA MOSER
JOHN O'DONOGHUE
STEPHANIE PADILLA
ANTHONY RILEY

I N D E X

P R O C E E D I N G S

Time: 9:10 a.m.

MS. SJOBLAD: Good morning, everyone. I'm Beverly Sjoblad from the Office of Pesticide Programs. We would like to welcome each and every one of you to the symposium on the neurotoxicity of mixtures, jointly sponsored by the OPP Technical Training Committee and the OPP Scientific and Technical Training Committee.

A full day has been scheduled. So without delay, I'd like to begin our program by introducing Dr. Steven Galson, Director of the OPP-TF Office of Science, Coordination and Policy. Dr. Galson.

DR. GALSON: Thank you very much, and I wanted to welcome all of you to the symposium on neurotoxicity of mixtures. I wanted to particularly thank the organizers in the Technical Training Committee of OPPT and the Scientific and Technical Training Committee in OPP, particularly Debbie Norris, Beverly Sjoblad, Trish Coleman, Ethel Brown and John Blowen.

It's a very important event to get this group of people together. We need to do it more, and I thank you for your efforts to bring us here together on this important topic.

I also want to thank the speakers who are here from industry, from university centers, and give a particular thanks to Herman Koeter for leaving the springtime in Paris. Where are you, Herman? It's probably raining there, too, but Herman is truly one of the most patient people in the continent of Europe, I'm convinced, and we're very grateful to him for the important work that he is doing on harmonizing guidelines in the OECD.

These guidelines are becoming increasingly important as the world and the chemical regulation world becomes more globalized, and he's really the one who facilitates the involvement of so many EPA staff and American government officials and scientists in this important work. So thank you, Herman.

Why is this symposium important? For the first thing, it's sponsorship. These training committees that got together to provide this opportunity are doing work that we don't have enough of. There isn't enough education and career development for the scientists in our part of the agency.

As you probably know, we're the largest group of scientists in EPA outside of the Office of Research and Development, and it's critically

important for us to get together as a group to see each other's faces and get to talk about issues, particularly pressing issues, relevant issues such as this.

The second major reason this is important is that neurotoxicity is a very, very critical scientific issue. It's received more and more focus over the last few decades with the realization that even small amounts of neurotoxins, particularly during sensitive periods in infancy and childhood and fetal life, can result in profoundly deleterious effects.

It's very important that we get risk assessment for neurotoxins right and that we keep up with the state of the science. So the relevancy of this topic is really, really perfect.

The other -- third reason that it's important is that the adjectives that were placed on the title, the analysis of mixtures, the analysis of cumulative risks, aggregate risks, these are all areas that, I think we would all agree, are very important, that we don't know enough about. We don't have enough focus.

We know that multiple simultaneous exposures to neurotoxins clearly have different effects than exposure to those chemicals alone. Yet

we don't have good methods to do risk assessment in these areas.

Under the Food Quality Protection Act mandate that OPPTS received, we have really gone farther than anybody else in the country and anybody else in the world on developing these kind of risk assessment methods and guidelines. So it's really important that we talk about this and that everybody is aware of what's going on.

We are really on the cutting edge in this area. So it's, again, a perfect reason to be here.

So I look forward to hearing at least some of the day today, and I wish all of you a good, thoughtful learning experience, and I'm really happy to be here and happy that we are doing it. Thanks.

DR. NORRIS: Thank you, Dr. Galson, and thank you all for being here today. Welcome and good morning.

First, I must tell you that today at our symposium on the neurotoxicity of chemical mixtures, we're not here in any way to review or establish EPA policy. We are here rather for the pleasure and opportunity of scientific exchange, which means that we can discuss scientific concepts, ideas, and data for the sheer joy of it, and it is a great joy to me

to do that.

As a self-proclaimed expert on the brain, I was talking yesterday with a self-proclaimed expert on the human mind, Dr. Tara Brock. Dr. Tara Brock told me that the mind has about 80,000 thoughts a day. Knowing the brain and how it functions, I added another thought to my cumulative total for the day, and that was that the brain probably processes even more than that each day.

Then Dr. Brock added that 98 percent of these thoughts we have each day are ones we've probably already had. I hope to change those statistics today. I think that we will greatly broaden our concept of the science of neurotoxicology and the neurotoxicology of mixtures.

Today we have the opportunity to hear from some of the world's experts in neurotoxicology and in the regulatory guidelines that we use and consider in evaluating toxicity. I have the pleasure of introducing to you this morning's speakers, and I will introduce them all to you now and then briefly one at a time as they speak:

Dr. John O'Donoghue who is Director of the health and Environmental Laboratories for Eastman Kodak Company, and I know him as the editor of several

volumes on the neurotoxicity of industrial chemicals which I use as references frequently.

Dr. Ginger Moser, who is a neurotoxicologist from the Neurotoxicology Division of EPA's laboratories in RTP. Ginger has received EPA's highest scientific achievement award for her work on EPA test methods and guidelines.

Dr. Stephanie Padilla is Chief of the Cellular and Molecular Toxicology Branch, and she is also from the Neurotoxicology Division of EPA's laboratories at RTP.

Dr. Will Boyes -- have you arrived yet? No. The last I heard, Dr. Boyes' plane had landed in Baltimore, and due to the weather or due to some unforeseen event. So I've asked -- If Dr. Boyes doesn't arrive, in an effort to stay on schedule, I've asked Dr. Abou-Donia if he would come forward to this morning's talks, in case that happens.

Dr. Abou-Donia is Professor of Pharmacology and Cancer and Neurobiology at the Duke University Medical Center.

Dr. Greg Christoph, who I'm so happy you're here with us today, because he is retiring tomorrow as head of Neurotoxicology at DuPont Company.

So we are very lucky and delighted to have

these speakers with us this morning. Thank you, and I will let Dr. O'Donoghue begin our program.

DR. O'DONOGHUE: Good morning. I'm very happy to be here. I have a little bit of trepidation, because every time I've been moving my digital slides between PCs, things have changed, and nothing has migrated since the last change.

As Debbie said, I'm John O'Donoghue with Eastman Kodak. What I'd like to talk to you about this morning is how we integrate neurotoxicology into our pollution prevention program.

My company is not in the pesticide business. So we are not really going to talk very much about OPs or pesticides, but rather some relatively common and mundane materials. Our focus is oftentimes, when we find a material that has neurotoxic properties, is to find what the limits of the effect are. In many cases, it's to eliminate it from the supply chain.

So in many cases, we are not trying to do a quantitative assessment. We are trying to think through the process of identifying materials, identifying safe levels, and elimination through a pollution prevention project.

Now the types of materials I want to talk

about are constituents of relatively common materials, paints, adhesives, inks and greases. We use a lot of different mixtures in our everyday activity, and what we are concerned about is how these materials might interact.

This is just an overhead of what we would consider the manufacturing process to be. So within the box you actually have the manufacturing process. Then what I've done highlighted in gold are those aspects of the process where we are looking at assessing chemicals.

So the process isn't just to look at products or co-products. We are looking at raw materials that come in. We are looking at the manufacturing process, whether heat is supplied to those materials, whether they become aerosolized, how they are used, and if the product is to be reused or recycled, is there anything in the product that is going to be potential during the recycling process. Then we look at what are our potential waste due to air emissions, water emissions or other types of releases.

The main thing we don't look at is on the left, which is kind of earth, wind and fire, which is going back to actually the mines and looking at some

of the raw materials. So we are looking at lots of different types of materials used in lots of different ways.

Now the process we use is a fairly straightforward process, and it actually has a lot of similarities to the process that's used for assessing materials under TSCA. One of the things we look at very early on is analogous materials. Do we have chemicals in our historical toxicology file that can tell us something about a new material or how a material might interact with the mixture.

We look at exposure, production, and how people are going to use the materials. Based on that, we do a preliminary chemical evaluation. We decide at that point if there is data that indicates there is interaction or there is neurotoxicity. We may eliminate the material at that point.

If not, then the material moves on to what we call a testing strategy. We decide what types of tests we might run on those materials, conduct the test, evaluate it, and then cycle back through.

We also have programs to look at what I've put down as process control and health assessments. We have industrial hygiene data available to us. We have employee medical information, not for the

individual employees but for groups of employees, and we also operate an 800 number for customers or consumers, if they have a question.

So this is part of the process. If we were to detect something post-marketplace, we have a process then to go back in and reevaluate the material.

Now one of the interesting -- or latest aspects of this is we've been working with Bill Law from your office to integrate the pollution prevention framework into our assessment process. This doesn't have specific aspects of it that are related to neurotoxicity, but there are some general pieces of information that are in the pollution prevention software that we integrate into our process.

So, for example, we are able to estimate chemical and physical properties of the materials based on structure activity relationships. We look at housing and the environment, and how was this material apt to be transformed, and is it going to be transformed into something which is a neurotoxin; and we look particularly at cancer hazard potential. Is there something about the structure of this chemical that would have some alerts from the point of view of producing cancer in the nervous system?

So this has become part of our overall assessment process. Now the types of effects that we're talking about fall in a variety of different categories. Chemicals can have a number of potential biological effects. Some of them are relatively direct in that they can cause irritation to sensory organs, irritation to the eyes, to the nose.

There can be some general C&S toxicity due to effects on, say, the liver or the kidney. Then there are some specific effects that chemicals have on the nervous system.

When we're talking about the mixtures, I'm talking primarily about pharmacologic and neuro-degenerative effects that chemicals or chemical mixtures can have, not the indirect effects through C&S depression or irritation.

There are also potentially effects that are neuro-oncologic effects that can be caused by chemicals. These are relatively rare, but it is something that has to be considered in assessing a new chemical.

Now the possible relationships that materials have to each other with regard to neurotoxicity are not that different from other organ systems, in that we are concerned about if you have

two materials that have the same effect, they both produce peripheral neuropathy, if there is a combined exposure, is that effect additive?

Are there synergistic effects in that two chemicals will have the same effect, but when a co-exposure occurs, the effect is much greater than what the additivity effect would be.

Potentiation is a situation where we have a mixture which contains a neurotoxin and other materials which are not neurotoxic, and whether or not those materials enhance the neurotoxicity of the main agent.

Then inhibition: Although we see inhibition less commonly than some of the other effects, we do see materials that, in fact, inhibit neurotoxicity.

Now what I've done on this slide is put together some factors that affect how chemicals interact in a mixture situation that we have to be concerned about. The first is the rate and extent of some of the pharmacokinetic properties of the material.

For example, absorption: If you've got a mixture and you've got two neurotoxic materials, if they are absorbed at different rates, in fact, the

effects may not impact on each other. They may not be additive or synergistic.

For example, hydroquinone, which is a common black and white developer, at high doses has effects on the nervous system that cause tremor and convulsions if the dose is high enough. However, the material is very water soluble. It's absorbed very rapidly. The effects are produced within about 15 minutes, and the material is excreted with a half-time of about 17 minutes.

So if you have a mixture that has two materials, one is absorbed rapidly, metabolized and excreted, and the other material is not absorbed as quickly, what may happen is, yes, the effects on paper could be additive, but in fact they are not, because of the pharmacokinetic parameters.

That's also true of metabolism, the rates of metabolism. It could very well be that two materials have exactly the same effect, but they produce that effect through a mechanism which requires metabolism to a common metabolite, and if the extent or the rate of that metabolism isn't similar with the two materials, there may not be an interaction.

Another factor which has an impact is the exposure route. We have seen materials like 3-

heptanone. 3-heptanone is a material which is present in many foods as a flavoring agent. It's in perfumes, and we've looked at 3-heptanone from the point of view of trying to produce neurotoxicity with it, because if you look at the structure, it ought to be metabolized to a common neurotoxic gamma diketone.

In fact, with 3-heptanone what you find is that, by inhalation, you can't get enough material into the body to produce the gamma diketone which would result in a neurotoxicity. However, if you gavage the animals, you can produce the effect. The reason we think this is the case is that, by giving a very large bolus dose, we're increasing the likelihood of a first pass metabolic effect in the liver that results in neurotoxicity.

So if you are assessing the material, and the data you have is oral, it may not be relevant to the inhalation situation or vice versa. You may find that, if you test it by inhalation, you see no neurotoxicity. You test it by the oral route, and you do.

Mechanisms of action: Common mechanisms of action are obviously situations in which additivity is likely to occur. I put down -- The last issue is the blood-brain barrier, because there are a couple of

aspects of this which are important.

One is that some materials can damage the blood-brain barrier. So you may have a material which, when tested alone is not neurotoxic, but if tested with a material which somehow or other alters the blood-brain barrier, it would allow that material to get into the brain and, therefore, produce neurotoxicity.

Now there are also some slight differences in various species of animals that tell us that there are conditions where the blood-brain barrier is a limiting factor in neurotoxicity; for example, iramectin.

Iramectin is a parasiticide, and it's been -- It can be used in most strains of dogs without producing any neurotoxicity, but in some of the smaller collie breeds the blood-brain barrier is not as effective as other brains of dogs. What you end up seeing is neurotoxicity in those dogs.

It's the same material with the different breeds, but in fact, because there's a defect in the blood-brain barrier in one breed versus the other, you are seeing the effect. So there is a concern about whether the chemicals would alter the blood-brain barrier.

Now what I've done is I have put together some scenarios for materials that we've seen and had some concern about how they might interact with regard to a mixture. So the first one, I think, is a relatively simple scenario.

That is that we have chemicals which are similar. They have the same metabolites, and they have the same molecular target. So we would expect here that the effects that we would see would be of an additive nature.

An example of these materials is shown on this slide. Now on the upper lefthand corner is a chemical diagram of hexane. In the upper righthand corner there is a diagram of methyl N-butyl ketone. These materials are similar except for the carbonyl.

In the body both of these materials are metabolized to common metabolites. In fact, N-hexane can be metabolized to methyl N-butyl ketone. So that we end up with a common metabolite, 2,5-hexanedione; and a 2,5-hexanedione itself is the most neurotoxic of these metabolites.

Now we have tested these materials in rats and looked at them from a comparative point of view. On this slide what I have is the first line shows the methyl N-butyl ketone tested at 6.6 millimolar.

Now in comparing these materials, we use millimolar doses as compared to milligram per kilogram doses, because the body -- These chemical reactions occur on a molar basis, not on a milligram per kilogram basis. So doing the comparisons, it makes a lot more sense to use a millimolar basis for this.

What we have here is rats with neuropathy. That is, they are showing some clinical sign, some sort of weakness or some sort of sensory change or they have some histologic evidence in their peripheral or central nervous system.

Then we listed the number of days it took for those animals to reach the endpoint, and the endpoint in this case was that at least one of the legs of the animals was paralyzed by the material. So it's a pretty severe endpoint, but it's one that doesn't change very much.

What you find with these materials is that, as the animals are dosed, the clinical state varies slightly from day to day. So it's hard to pinpoint a point in time when you can say the effect is quite similar. By choosing this endpoint, which is stable, we can do a better comparison.

Then we've calculated what I refer to here as a neurotoxic index. This neurotoxic index is based

on the number of days that it took for the MNBk animals to be paralyzed. So because it took 55.8 days, any group that had its endpoint at that same time would have a neurotoxic index of one. Any group that took less time would have an index of greater than one or be more neurotoxic. So it's a basis for comparing the relative effects here.

Now with these two materials, you might expect that the effect would be additive, but we also dosed these animals with an equi-molar dose of N-hexane, and what you find out with N-hexane is you see no neurotoxicity with this material.

Now these dose levels correspond on a milligram per kilogram basis to somewhere around 600-700 milligrams per kilogram. So the N-hexane dose here is quite a substantial dose. It's not a minor dose but, in fact, there is -- Even though the metabolites are the same and you're giving the same molar amount of the material, the effect you're going to get here is not additive.

Now we doubled the dose of N-hexane to 13.2 millimolar, and that wasn't effective. Then we raised it up to 46.2, and 46.2 is as much material as we could get into the animal. That was the physical limitation here. It's about four grams per kilogram

of the material.

When you give that dose, you do get clinical effects. Here we got three of the five animals had effects, and four of the five had histologic effects; but it took almost twice as much time as with MNBk. So the effects here -- You have the same metabolites. The effects are not additive.

We also looked at a material called practical grade hexane. Practice grade hexane is actually what most people are familiar with. This is actually a mixture of hexanes. It's about 40 percent N-hexane. It has cyclohexane in it, methylpentane in it, and a variety of other materials.

Our original concern with this mixture is we were concerned that this mixture would, in fact, increase metabolism of N-hexane and be more neurotoxic. In fact, what we found was that the neurotoxicity was no more than with hexane alone. So these mixtures of hexanes didn't have any interaction.

Now there is another way to look at these materials. Hexane, MNBk, 2-hexanol and 2,5-hexanedione are all commercial -- have been commercial materials. N-butyl ketone now, there's a significant new use rule on, and 2,5-hexanedione itself has had relatively minor use. But in fact, these materials

could be used in combinations in some products.

So we looked at how we would go about trying to compare these materials and how to figure out on a relatively simple basis how to judge the relative activity of these materials if they are combined.

Now this data is very similar to the data that I showed you before on the neurotoxic index, in that for each of these materials we gave them the same millimolar dose, and we then looked at the onset of neurotoxicity. We divided that by the time it took for the MNBk animals.

So MNBk here has a neurotoxic index of three. N-hexane, because it took a higher dose and a much longer period of time to create neurotoxicity, has a neurotoxic index of -- I think it was less than .1. Then 2,5-hexanedione, which is the last material at the top of the screen, it took a third less time for the animals to develop neurotoxicity. So, in fact, it was three times more neurotoxic than hexanedione.

The other part of this relationship is the serum concentration of 2,5-hexanedione, because you can see that there is a relationship between how the neurotoxic index in the peak concentration -- the more

2,5-hexanedione was formed by these materials, the more neurotoxic it was.

So this allowed us a basis for actually combining these exposures and for looking at exposures to other materials, because we now knew that the effect was based on the serum 2,5-hexanedione level. We could measure that in blood. We could measure it in serum, and we could measure it in urine.

Therefore, it became a convenient biomarker of exposure, and also a convenient biomarker of effect, because you can actually quantitate at what level in the animals you will see neurotoxicity based on a 2,5-hexanedione level.

This parameter is one that's recommended now by ACGIH as a biological index for exposure to hexane and related materials. So it is possible to calculate how these similar materials are going to add together, but they don't add together in a simple fashion. It's not a matter of adding one mole of hexane to one mole of methyl N-butyl ketone and coming up with two. It's a matter of understanding what the metabolic pathways are and how they can be combined based on that common pathway.

Now the second scenario I put together is one in which there are similar classes of chemicals,

and in this particular case there are ketones and other similar alkane solvents that have different metabolites. They are not metabolized in the same way, but there are metabolic interactions that occur with the mixture.

This is a mixture that was proposed for use in paints and for plastics. This mixture was sent to us for evaluation, because there was a low level of MNBk in it. It's less than one percent MNBk. Our first thought with this was, well, that's probably not going to be enough to cause the solvent to be neurotoxic. There is also some 2-heptanone in it, which was a little bit of concern, because there are, at least in theory, ways in which 2-heptanone could form a gamma diketone.

So our initial read on this mixture was this probably isn't a problem, but we did some screening level tests on it and found that one of the components, 5-nonanone which was in there at 12 percent, when we actually tested the mixture, we found that 5-nonanone was metabolized to methyl N-butyl ketone.

I haven't shown you the whole pathway here. The methyl N-butyl ketone then is metabolized to 2,5-hexanedione. So the potential pathway here

between this and MNBk is very similar. The biochemistry is very similar except in this particular case we're getting decarboxylation, which makes the material a lot more neurotoxic than you might expect otherwise.

So before we had discovered this, we started off looking at the components of the mixture. This table shows again the chemicals that we looked at and the mixtures on the left, the day of onset of neuropathy, the number of days it took before we saw any evidence of clinical neuropathy, the most severe clinical neuropathy, and a +3 here is the animal is dragging one feet. A +1 is that the animal doesn't completely extend the foot, and a +2 indicates that the animal is having some problems with placing the foot. They tend to trip or misplace the foot, but they are able to walk.

Then the next column is the number of animals with clinical neuropathy at the end of 90 days, and the last column is the number of animals that had histologic changes at the end of 90 days.

So with the original material, this commercial grade methylheptylketone -- it was given at 2,000 milligrams per kilogram -- we got a neuropathy at about 60 days. It was severe, and all the animals

eventually had clinical signs or histopathology.

Now we also looked at the main ingredient of this material, which was 5-methyl-2-octanone, and we tested it at 2,000 milligrams per kilogram. In fact, we saw no effect with this material from a neurotoxicity point of view.

The other material that we looked at here was 5-nonanone. It's shown here as N-O-N, and the high dose that we used was 2,000 milligrams per kilogram, and we got neuropathy in 11 days. It was severe, and in this case the screening level animals all had it. They all had -- Both of them had clinical signs, and both of them had neurotoxic effects.

Now we dropped the dose down, and we got a similar effect. We dropped the dose down to 233 milligrams per kilogram. This equates to the amount of 5-nonanone that was in the original mixture. So the 12 percent 5-nonanone in the commercial methylheptylketone equates to 233 milligrams per kilogram, and here we saw no neuropathy in the animals, even though when we looked at them histologically there was a very slight neuropathy.

So there wasn't enough 5-nonanone in this mixture to account for the neurotoxicity that we were seeing. There wasn't enough MNBk in the mixture to

account for that or enough of the 2-heptanone.

So what we did is we went back, and we reconstituted the mixture with the two materials we had the greatest concern about, and that was 5-methyl-2-octanone and 5-nonanone. So we recreated the mixture and dosed the animals at 2,000 milligrams per kilogram.

In fact, what we saw was that we produced a neuropathy that was similar to the commercial mixture. All the animals had histologic changes, but they didn't all have clinical signs. So by combining the two materials, we got an effect that was similar to the mixture, but wasn't even quite as severe as the mixture. It was slightly less.

So there are other components in this mixture that are probably contributing to this. Probably the MNBk and the 2-heptanone are adding -- have an additive effect, but the effect that we're seeing here is a promotion of the metabolism of 5-nonanone to its ultimate neurotoxic endpoint, which is again a diketone.

Now because 5-methyl-2-octanone would do this, we were concerned about other mixtures. Having studied other mixtures of ketones, we were aware that methylethylketone, which is a very common solvent --

methylethylketone will potentiate the neurotoxicity of methyl N-butyl ketone. It enhances the production at 2.5 hexanedione.

So our thought here was, well, gee, would methylethylketone potentiate the effects of 5-nonanone. In fact, what we found, it didn't. We dosed these animals in a comparable way to the animals that got 5-methyl-2-octanone, and it didn't.

The reason we believe this is the case is because what the 5-methyl-2-octanone is doing is not promoting the metabolism of MNBk, but it's promoting the metabolism of 5-nonanone to MNBk. It's affecting the decarboxylation reaction but not the oxidation of 2,5-hexanedione; and because MEK doesn't do that, you don't see the potentiation in this particular case.

Now this is a summary of a number of the mixture studies that we've done. We've looked at MEK, and it potentiates N-hexane. It potentiates methyl N-butyl ketone. It does not potentiate 5-nonanone.

We've looked at 5-methyl-2-octanone which potentiates the effect of 5-nonanone, but it doesn't potentiate the effects of another material which produces a gamma diketone, which is ethyl N-butylketone.

Others have looked at the effects of

toluene, and toluene, in fact, inhibits the neurotoxicity of N-hexane. This is an interesting inhibition, because I'm sure Dr. Boyes is going to talk about toluene. Toluene itself is neurotoxic. It produces auditory problems.

Here you have two neurotoxic materials, one that produces an auditory change, N-hexane which produces a peripheral neuropathy. You give them together, and you don't get the peripheral neuropathy. So you might think that, because they are neurotoxic, they would have some kind of interaction. In fact, they do, but it's the opposite of what you would actually expect.

Now this scenario I put together involves significantly different chemicals. On a molecular basis, the chemicals are very different. They have different metabolites. They have a different molecular target. They don't operate by exactly the same mechanism. Yet they produce the same effect in the animal.

I'm sure later in the day people are going to talk about organophosphates. I put this cartoon up here in case you're not familiar with the neuromuscular junction. This is a cartoon of the neuromuscular junction.

This is the presynaptic area. This is the synaptic cleft and the postsynaptic area. Now what happens here is the action potential comes down. You get calcium influx into the neuromuscular junction. You get fusion of the synaptic vesicles with the presynaptic membrane and release of acetylcholine into the presynaptic cleft, and then you get binding on the postsynaptic membrane, and you get an action potential in the muscle. The muscle contracts.

Now what causes this to reverse is acetylcholinerase, which breaks down the acetylcholine and reduces its effect on the postsynaptic membrane. Now these membranes then are -- These vesicles then are recycled back to the cell body.

Now with this next material, this is 4-nitropyridine-n-oxide. It was an R&D material that people were pretty excited about, and we were concerned about it, because pyridines can have neurotoxic effects.

So we did some screening level studies with this material, and this is the acute toxicity data that we got from this material, which caused us to be very concerned. You see the dermal LD_{50}? The dermal LD_{50} is a gram per kilogram. The oral LD_{50} is 50-100.

Now if you put it in the eye, and the eye is unwashed, the LD_{50} here is greater than 10 milligrams per kilogram. You, in fact, see clinical effects in the animals, but none of the animals die. The effects that you start to see are muscle twitching, and then you see massive vesiculation in the skeletal muscle, seizures, and then if the animals get a high enough dose of this material, they stop breathing and die.

What you're seeing is an acute cholinergic effect in these animals due to application to the eye. Now if you put this material in the eye and you wash it out, it has an LD_{50}, we say, between one and ten. We've never actually been able to calculate precisely what it is, but we've had animals die with one milligram per kilogram of this material in the eye.

We think what happens is that, when you go to wash it out, what you in fact do is solubilize it, and it's very rapidly absorbed through the eye and into the CNS.

Now this material, which is a test material on the left, is very similar to 4-aminopyridine. These pyradines are used pharmacologically to enhance the release of acetylcholine. People that have Eaton-Lambert

syndrome, for example, have very weak muscle activity, and one way in which to enhance the activity is to provide them 4-aminopyridine.

4-aminopyridine affects the potassium pore in the membrane. What happens is the action potential, when it comes down to the endplate, is prolonged. So you get a long action potential, and the prolongation of the action potential allows release of significantly more acetylcholine.

So what you end up with is a cholinergic crisis. Now our concern with these materials, if they are in a mixture with a material which is also a cholinesterase inhibitor, you would have two materials that produce a cholinergic crisis by two different mechanisms. In one case it enhances the action potential. It releases more acetylcholine. In the other case, it inhibits the breakdown of that acetylcholine.

So you get an enhancement of the effect. This enhancement occurs by two different -- at two different molecular targets. The effect in the animal is similar, and the effects are additive, even though the mechanism of action isn't additive.

Now the fourth scenario I put down here are we have significantly different chemicals. They

have different metabolites, but they have the same molecular target.

This cartoon is to help you understand some of the neurobiology associated with materials that we're concerned with here. The axon is the mechanism -- the pathway by which the cell body communicates with the periphery, whatever that periphery happens to be, whether it happens to a neuromuscular junction or happens to be a sensory endpoint.

There is movement of some relatively large structures. The organelles that are formed in the cell body are transported to the neuromuscular junction. So the synaptic vesicles, for example, are moved from the cell body to the endplate and back again.

This cellular movement is very important to the life of that axon. If there is anything that interferes with that intracellular movement, you, in fact, get some form of axonopathy. The materials that we have been concerned with have been materials such as acrylamide, such as MNBk, such as N-hexane.

We've looked at the mechanism of action of these materials. This schematic is a schematic I put together about how acrylamide, in fact, can result in

a peripheral neuropathy. In this particular case, the acrylamide is absorbed and distributed.

We know it undergoes hepatic metabolism. We're not totally sure whether the metabolites in acrylamide itself can have a neurotoxic effect, but we know that from binding acrylamide to various targets in the axon you get interference with axonal transport. Then you get some degree of axonal degradation.

Now depending on the dose level, you actually also get axonal regeneration. So there is a balance here between very low levels of exposure axons being damaged and axons regenerating. If, in fact, the damage occurs at a faster rate than regeneration, you get clinical deficits.

Now what we've been concerned about is that these materials all have some very common similarities in that they all have effects on glycolytic enzymes. They have effects on glycolytic enzymes which has an impact on energy metabolism in the axon and can affect axonal transport.

They have effects on the microtrabeculae system in that they tend to stabilize the axonal proteins, and the stabilization can result in reduced axonal flow, and that will slow down transport.

They can also interact with the active transport proteins, and they can interfere with movement, having an impact on retrograde flow, and in the end they all result in this dying back neuropathy. They cause a neuropathy by impacting axonal flow.

So our concern has been that there is a common mechanism of action. Even though these chemicals are very different, there is no reason that we see that these materials should not interact and cause peripheral neuropathy at -- I won't say low doses, but our concern has been that, if we give subneurotoxic doses of some of these materials, are those effects additive?

From what we have seen, we think they probably could be additive, although for the most part, we have not had a reason to actually create such mixtures.

So in summary, there are interactions among chemicals that are affecting neurotoxicity. These interactions are modulated by pharmacokinetic parameters, absorption, metabolism excretion. There are metabolic interactions that occur outside of the nervous system -- we see them primarily in the liver -- that affect neurotoxicity.

There are also nervous system specific

vulnerabilities, either with the blood-brain barrier or with the transport system in the nervous system, that offer unique opportunities for interactions of chemicals.

We see mechanisms of action that are common for a number of neurotoxic chemicals, and thus offer the opportunity for an additivity effect.

So in summary we can increase or decrease the likelihood of observing neurotoxicity with a particular mixture through any of these various parameters. Thank you. Do you want to take questions now, Debbie, or later? Questions?

(Applause.)

(INAUDIBLE QUESTION)

DR. O'DONOGHUE: The way we've determined additive effects is to go back in and reconstitute the mixture at various concentrations and try to see if we can reproduce that additive effect with the mixture. That's what we tried to do with some of the ketone solvents.

(INAUDIBLE QUESTION)

MR. O'DONOGHUE: No. We tried to -- He was asking how we went about doing the additive studies. What we actually do is try to understand what the chemical mixture is and then recreate that

mixture and then retest the mixture. We are not doing it by a modeling program.

Does that answer your question?

I think, actually, when you talk about cholinesterase inhibition later on, we'll probably talk more about how those things are actually modeled. The reason we don't do a lot of that is because when we find one of these materials, our main objective is, frankly, to get rid of it. It's not to figure out a way to use it.

We are not intentionally using neurotoxic materials. We're trying to understand what the limits of their safe use are and, in many cases, get rid of the materials, if possible. So our goal is quite a bit different.

DR. MOSER: I wanted to thank Debbie and the organizers for inviting me to be here today. I appreciate this opportunity.

I'm going to switch gears a little bit from what John was covering, and I'm going to talk more specifically about one study and also get into some of the more statistical analysis on that study.

As we all know, and the reason that we are all here, I think, is that most environmental chemical exposures do occur in multiple. So we are not exposed

to just single chemicals. So a lot of the information that we have about single chemicals may not be as appropriate to understanding mixtures.

For now, there is just simply no way of predicting when or what kind of interaction may be produced when there is exposure to multiple chemicals, and trying to model these interactions in the laboratory requires a lot of different types of considerations, and also the statistical analysis of these interactions becomes very difficult very quickly.

What I want to present here today is the data from a large, rather a huge study that we've conducted quite a few years back now. I want to try to present some data that you may find interesting on the mixture, and I also want to try to illustrate the complexity of this type of study, both in terms of its statistical analysis and the data interpretation.

In this particular study we wanted to go past binary combinations, which are typically the way that people look at chemical mixtures in the laboratory. We wanted to go all the way from two to three chemicals.

The project was initiated as part of a Superfund project. We wanted to study chemicals that

were frequently found in Superfund sites and were found to co-occur.

We used a full factorial design to study these interactions of these three chemicals, which means we had three chemicals and five dose levels of each chemical. One of those dose levels was zero, and then there were four other dose levels.

We looked at all possible combinations. so it was a five to the three study. Five to the three gives you 125 treatment groups. In the study we used ten rats for each treatment. So you can see why I say it's such a huge study.

The study was actually conducted in collaboration with some other people at EPA, and we did neurotoxicological evaluations on the animals. In the same animals we did general toxicity studies, and we also at the end of the study took organ -- did organ histopathology and clinical chemistries.

The study was run in conjunction with the developmental toxicity study using the same chemicals and similar dose levels. The results of the developmental tox study have been published already, but this is the debut for the neurotox data.

I'm going to briefly describe what response surface modeling is. It's a method that aims

to mathematically describe the surface of the response, the response to the combination of chemicals, all combinations of chemicals.

It uses a factorial design where chemicals are present -- all the different chemicals are present in different levels, dose levels. Some of the advantages are that it does use all the experimental data. There is no mathematically derived numbers like ED_{50}.

It does estimate the response surface over a range of doses. It provides estimates for both the individual chemical as well as the interactions between the chemicals. Theoretically, there is no limit to the number of chemicals that can be studied this way, even though you can only picture -- you know, draw a graph for combinations of two chemicals, mathematically you could have an infinite number of chemicals.

The other advantage to this type of modeling that we did was that there would be data available to take out subsets of the data, so that we could analyze the subsets of data and see whether we could predict the same result using a reduced dataset compared to the full dataset.

Now as I say, we did the

neurotoxicological evaluations in these animals. We used the functional observational battery or FOB, which probably most of you all are familiar with. It's a battery of tests that rapidly evaluates neurological function of the animals.

We also used motor activity using an automated assessment of locomotor activity. The FOB is a neurological exam. It does evaluate neuromuscular dysfunction, sensory deficits, autonomic changes, and changes in activity levels and reactivity or excitability of the animal.

The standardized series of open field and manipulative tests that are well standardized are used widely now to screen for hazard identification for potential neurotoxicity. So these were the tests that we used in this study.

Protocol here shows that we used Fischer-344 female rats. The reason for this was, as I said, we did this study in conjunction with the developmental tox study. So, of course, we had to use females. We used the Fischer-344, because that was the standard strain that's used in the Chernov-Kavlok assay.

We did neurobehavioral testing, as I've already said. We tested the animals before dosing

began, four hours after the first dose, 24 hours after the first dose. We dosed the animals repeatedly for ten days, and then we also tested them at four and 24 hours after that tenth dose.

Because of the number of treatment groups, obviously, we couldn't test all the animals at one time. We had to split up the study into replicates. We had the statistician draw up the replicates for us so that we had five replicates of 250 animals in each one. Of course, we can't test 250 animals at a time either. So we had to break down the replicates into blocks so that essentially we tested 50 animals at a time.

The chemicals that we chose to study are listed here. The first one is heptachlor. It's a cyclodiene pesticide that's very persistent in the environment. It does act on the nervous system. It blocks the GABA-ergic system. So it causes excitation.

The next chemical is diethylhexylphthalate or DEH. It's a liver toxicant, and it's not known to act on the nervous system at all.

Then the last one is trichloroethylene, which is a volatile organic solvent. The mechanism for trichloroethylene isn't exactly known, but it is

known as a general type of CNS depressant.

I want to point out that only two of these chemicals did act on the nervous system, and the mechanisms of actions are very different for the three chemicals.

The doses that we used are listed here. For heptachlor -- or for all the compounds that we tested, we chose the doses in order to span the range of effectiveness. We wanted to be sure that the high dose was effective and that the low dose had essentially no effects, and we wanted to be able to construct a good dose response curve with these four doses.

One thing to point out is that with the response surface analysis you don't need to have your dose basing be any specific way. So you can see that the dose basing was very different for the different compounds. We could select our doses in order to construct the best dose response curve. We were not restrained in any way.

The other thing to point out is that the components were mixed together into the dosing solution, so that the rats only received one dose a day instead of getting the three different doses. So we had basically at any one time 50 different doses

being given in a day, and they were all dissolved in corn oil, which was our vehicle.

Now for the data analysis. This was really the trickiest part of the whole study. The FOB produces different kinds of endpoints. Some of the data are binary or just simply yes/no data. Some of them are continuous like body weight or activity count, and some of them -- most of them, actually, are ordinal. They are ranked scores.

There are published methodologies for analyzing binary and continuous data using response surface analysis. So far, even now, there is no analysis that is developed for ordinal types of data. So for those data we had to make transformations before we could actually put the numbers into the response surface analysis.

With the data analysis we get parameters that will -- you can get parameters for an overall test of interactions. So it will test whether or not there is a significant deviation from additivity, significant less than probability value .05.

You can also get individual parameters for the effects of the chemicals by themselves. You can look at the binary combinations of each three chemicals. So you could see whether there is

interactions between just two of the chemicals, and then you can also look at the tertiary interaction or the three-way interaction.

We could also analyze -- Because of the way that we set up the replicates, we could also analyze whether there were replicate effects or not. We did do a reduced design -- a reduced analysis. What we did was take the zero level and the data from the next to the low dose and the high dose. So that the same type of analysis was run using just two doses of each compound instead of the four doses of each compound.

We were hoping that, if this was predictive of the full factorial design, this would be a more efficient way to do the study.

Then because of the time restraints, we had to limit the analysis to just one time point, and the time that we chose was the four-hour data after the tenth dose.

First, I'll go over the effects of the individual compounds. What's listed here are the -- The measures of the functional observational battery are listed here. As you can see, trichloroethylene, being a CNS depressant, does tend to decrease or depress most of the nervous system functions.

We get decreases in grip strength. We get gait changes, decreases in activity levels. Kind of interesting, we did get increases in alertness, but basically decreases on most of the other responsiveness tests like sensory motor responses.

We saw some general health changes with piloerection and lacrimation, and we saw body weight decreases and a small amount of lethality at the high dose.

Heptachlor, as I've already said, inhibits the inhibitory transmission. So it actually produces increases in some of the nervous system functions. We get increases in grip strength, gait changes again. We do see decreases in locomotor activity with heptachlor and these types of chemicals, but we get increases in responsiveness to both being handled and some of the sensory tests.

We also got lacrimation, some salivation, decreases in body weight, and a small amount of lethality. As I've already said, DEHP does not act on the nervous system, and we got absolutely no effects on these tests with that chemical by itself.

This is the results of the statistical analysis. There is actually a lot on this slide. So I may need to take a minute.

Again, these are the endpoints that we tested. This first column is just whether or not there was a significant interaction or significant deviation for additivity. As you can see, for almost all the endpoints there was a significant interaction. In fact, just about every single one of them showed a significant interaction.

Now what's listed here is the algebraic sign of the parameters, where there was a significant parameter. So that means that, if the parameter is significantly different from additivity, the algebraic sign is listed.

What that means is a positive sign shows that the effect is increasing with dose. So you have a positive going to this response curve, and a negative sign is a decreasing dose response curve.

So for instance, with hind limb grip strength we decreased grip strength with TCE by itself, and we increased grip strength with heptachlor by itself. Now in cases like gait score -- I want to point this one out -- we did see gait changes with both TCE and heptachlor. So we had a positive parameter for both of those, but then the interaction, the trichloroethylene by heptachlor interaction had a negative parameter.

That means that the dose response curve is either decreasing or else it's not increasing as much, and that's interpreted as being antagonism. So whenever the sign is opposite from the signs of the individual parameters, that's antagonism; whereas, if it was the same direction, then that would be considered synergism, because the effect would be greater than the two compounds alone.

As you can see, for most of these characterizations we did have antagonism. Interestingly, with lethality we did see significant effects of both individual compounds. When you looked at trichloroethylene and heptachlor together, we had antagonism.

We also had antagonism with the DEHP and the heptachlor as a binary combination. Remember that DEHP had no effect on its own. But then if you look at the three-way interaction, the three chemicals together actually produce synergism.

So this was kind of the worst case scenario where for most of the neurological endpoints we got antagonism, and then for lethality, which is probably the most important toxicity, we got synergism.

Then we have instances that we can't

really characterize what kind of interaction it was, because none of these interaction parameters were significant. So you couldn't really say whether it was antagonism or synergism, and that actually occurred for half of the endpoints where we got effects.

So we compared the results of the overall analysis, the full factorial design, with the data from the reduced analysis. So this is just the same column that I just showed with the number of yeses and noes, showing whether or not there was a statistically significant interaction, and then compared to the reduced dataset analysis.

For the most part, they did not match up. In fact, there were very, very few cases where they did give the same result. So it's obvious that, looking at just the two doses the way that we did, you could not predict the outcome that you would have gotten when you looked at the full dose response curves.

So now I'm going to try to show a little bit of the data. This is the data from gait score. Gait score is a subjective assessment of the abnormality of gait by the animals moving around in an open field. It's scored from one to four, with one

being normal and four being the most severe abnormality.

What I've got plotted here are the individual compounds, and the circles indicate the average gait score. So this is the average rank. You can see, with the TCE and the heptachlor, that there is an increasing severity of gait.

Then the triangles indicate incidence or the number of rats that show an abnormal gait. What we had to do, because there is no statistical model to handle rank data, we had to convert all the data to incidence. So either the animals had abnormal gait or they didn't.

So you can see what the dose response curves look like for the incidence for trichloroethylene alone, heptachlor, and DEHP basically didn't have any effect on gait.

So the first place to look at is to start looking at the binary combinations. One of the best ways to look at binary combinations is to construct an isobologram. What you do is you construct dose response curves for, say, chemical A in the presence of many different doses of the other chemical or chemical B.

You can actually then derive ED_{50} for

chemical A, and there will be different ED_{50}, depending on the dose, of B -- depending on what dose B was. Then you can plot it.

So this line shows the ED_{50} for chemical A, the ED_{50} for chemical B by itself. Just draw a line, and that's the theoretical line of additivity. If you give a dose, say, of four of chemical B and then do a dose response with chemical A, if the ED_{50} falls right about here, then it falls on the line of additivity, and that's taken to be a statistical -- That's interpreted to be -- Statistically, that's interpreted to be additivity. But if the ED_{50} is much less, it falls in this area which is synergism, and if it's much higher than the ED_{50} would have been, it would be antagonism.

This is just a standard way of looking at binary combinations. Then this shows the analysis with gait score. What I did was calculate the ED_{50} for heptachlor alone, which was about 14, ED_{50} for trichloroethylene alone, which was about 1,000 -- these are milligrams per kilogram -- and connected the line, and then looked at the dose response for heptachlor in the presence of all the different doses of trichloroethylene and the dose response for trichlorethylene in the presence of all the doses of

heptachlor.

That's what these ED_{50} points are that are plotted. You can see that they all fall in the area of antagonism, and that was kind of a relief to me, because that's what the response surface analysis had said also, that there was antagonism between these two chemicals.

Now the problem with the isobolograms is that it doesn't show all the data. You have no idea what the dose response curves look like, and these ED_{50} are mathematically derived numbers.

So this 3-D graph actually shows all the data for the combination of heptachlor and trichloroethylene. Note that this all in the presence of zero dose of DEHP. So this is just a binary combination right now.

This kind of purple looking bar showed the dose response for trichloroethylene in the presence of zero heptachlor. So that's trichloroethylene alone, just like you saw a couple of graphs back. Then heptachlor alone is this set of bars right here.

Now if you, say, look at the high dose of trichloroethylene by itself and then you start adding heptachlor to it, you actually get less abnormal gaits, especially at this mid-dose of heptachlor.

That's where the antagonism is showing up.

In the same way, if you look at the high dose of heptachlor, you've got about 50 percent of the animals showing abnormal gait, but as you add low doses of trichloroethylene, that percentage drops down. Then even at the highest dose of the two combinations, the two chemicals in combination, you're not getting much more effect than you got with the individual compounds.

So this kind of dose response here where you see all the data, you can actually see where the antagonism is.

Now you can get into the really hairy data. This is the three-way interaction, and this is the data for lethality. As I said, there was a significant interaction between trichloroethylene and heptachlor, between DEHP and heptachlor, and also between all three compounds with lethality.

So this graph here is the same as what you saw with the gait score with a zero level of DEHP. As you can see, the chemicals by themselves produce very little lethality at the high doses. In the low dose range here, we've got low doses of trichloroethylene, the incidence of lethality in the heptachlor high dose was actually less. So you can see where the

antagonism is coming from.

Again, this is all dose dependent kinds of interactions, which makes interpretation even harder.

Now if DEHP had no effect on the interaction between trichloroethylene and heptachlor, then all five of these graphs would look the same or essentially the same. If you just kind of jump from one to the next, you can see that they don't look the same.

So we've got the low dose of DEHP. Then it goes up a dose, and then this is the highest dose. Now you recall I said that when you looked at the three chemicals together, you actually had synergism, and the best way of looking at that is to look at this high dose combination.

This is the highest level of DEHP, the highest dose of trichloroethylene and the highest dose of heptachlor, and we got 100 percent lethality in those animals. So this shows where the synergistic responses actually are.

So this just kind of summarizes the study. Basically, we had deviations from additivity in 77 percent of the endpoints. So a great number of the endpoints did show significant interactions.

The reduced dataset predicted this kind of

level of interactions for only three of the ten. So it was not very efficient in being predictive. The interactions mostly involved trichloroethylene and heptachlor, which kind of makes sense; because those are the two that act on the nervous system, in the first place. But some of the interactions did include DEHP, which has no effect on its own.

Some of the interactions were characterized as antagonism. Other ones could not be characterized. Then, of course, lethality showed synergistic effects, and some of these effects were dose dependent. The interactions were dose dependent.

So the bottom line is that the outcome could not be predicted on the basis of the functional effects of these chemicals, and they could not be predicted based on the mechanisms of action.

So, basically, there's just no substitute -- Based on these data, there's no substitute for experimental testing, if you're looking at compounds with different mechanisms of action.

Some of the lessons learned from the study is that these kinds of factorial designs are not practical, I don't think, for general laboratory testing. It took 1,250 rats to generate all these data, and it was about a year and a half to two years

to collect the data and then another year and a half to two years to actually analyze the data. So it's a tremendous drain on resources.

There's still analyses that could be run. There are other time points. As I said, we only analyzed that one time point. We actually should go back and analyze the data using the pre-dosing data as a covariate, because some of these endpoints, the response does depend on what the baseline data were.

Then there is still a method that needs to be developed to analyze the ranked data. Converting the ranked data to incidence data is probably not the best way, but it's the only way we have of doing it.

Then just to end, I need to acknowledge that this work was conducted under contract to EPA. The laboratory work was conducted by ManTech Environmental, and that was a contract to the HERL back when we were HERL back before we became a national lab.

Joe Elder and Bob Dyer were the contacts at the EPA and helped us a lot with setting up these studies and keeping them going, and Billie did most of the testing for the neurotox testing, and Mike did all the general tox testing as well as preparing all these 125 dosing solutions three times for each study. So

that was quite a feat in itself.

The data analysis were all conducted at the Medical College of Virginia under the supervision of Chris Gennings, and she had a graduate student, Carol, who did all this work and, I think, finished her whole thesis and probably killed herself after finishing all this analysis.

That's it. So if we have time, I'd like to answer some questions.

(Applause.)

DR. NORRIS: Can I remind people to please use the microphone if you have questions, and we have just a few minutes.

QUESTION: Could you speak a little bit more about some of the interactions you saw at the lower doses; you know, the very lowest doses that you tested.

DR. MOSER: For the other endpoints, you mean?

QUESTION: Yes.

DR. MOSER: Well, for the most part, if you actually graphed out all the data and looked at it, which generates millions of these 3-D plots, it looked pretty much the same kind of pattern that you saw with lethality and with gait score.

If you started adding low doses of the second chemical, the effects of the first chemical tend to go away. So it looked more like there was antagonism for most of the endpoints. Then when you start getting up to higher levels again, that goes away, and you start getting more effects.

It just is a clear case of the antagonism being dependent on the dose of the chemicals. I don't know if that's enough to address, but we don't have any other characterization of it besides that.

QUESTION: I wanted to ask you a question in terms of synergism of lethality. Number one, were the deaths very shortly after you gavaged them or were they more long term, and how many -- I actually have three questions. The second question is how long after the tenth day of dosing did you hold the animals or did you sacrifice them right after you did the neurological testing?

Third, in terms of the lethality issue, would the volume of the dose being important?

DR. MOSER: Okay. If I can remember all three, the first one was, no, we never got death immediately after dosing which, of course, is a very important thing when you're talking about this number of animals and gavaging. But the technicians that we

had doing this were very good and never did lung any animals.

The death actually occurred during the last couple of days of dosing, and we found and have shown with heptachlor other times, too, that when you dose heptachlor repeatedly, it tends to build up and you start to see toxicity after days of repeated dosing that you don't see on the first day.

So all the deaths occurred on about the seventh, eighth and ninth and tenth day of dosing, and usually what would happen is we would come in the next morning and find them dead. We rarely ever saw them die during the day.

Because the study was run with the general tox, what we did was we tested them at 24 hours after that tenth dose, and then immediately after that there were sacrificed and we took the organs out, and weighed the organs, prepared them for pathology and took blood for clinical chemistries and that sort of thing. So we never held them past that eleventh day.

Oh, the volume. I'm trying to remember. I think the volume that we used was 5 milliliters per kilogram, which is a very reasonable level for oral dosing. We had to kind of hold some of these doses down to make sure we could do that, but I think that's

what it was, 5 mls per kilogram.

Any other question? Thank you.

DR. NORRIS: Thank you, Ginger. I'm trying to be quick with questions at this point and stay on schedule. We will have time for more questions during the panel discussion.

Our next speaker is Dr. Stephanie Padilla. Thank you.

DR. PADILLA: Let's change gears yet again. This is a group of studies that Wendy Haines, who is the second author here, who is a graduate student in my laboratory, started about a year and a half ago. Basically, her doctoral dissertation is going to be looking at mixtures of OP pesticides, OPs and carbamates, and we'll probably also do an OP and a pyrithroid pesticide.

We first started out with these two compounds, chlorpyrifos and diazinon. I'm sure these structures are probably familiar to many people in the room. We chose them, because we thought we would get a more than additive interaction on the basis of their structures, their metabolism, and their mechanism of action.

They are both anticholinesterase

inhibitors or they are both anticholinesterase compounds. So they both inhibit acetylcholinesterase as their mechanism of action. They are both converted from their parent compound here. This sulphur is replaced by an oxygen, and this becomes chlorpyrifos oxone, and this one becomes diazoxone, which are very potent cholinesterase inhibitors.

This conversion takes place by the P-450 enzymes, mostly in the liver. So they could interact at that level. They are both detoxified via carboxylesterases, stoichiometric binding by the carboxylesterases, and they are also both substrates for the A-esterases and can be hydrolyzed by the A-esterases.

So their metabolism is extremely similar in the animal. What we wanted to do was not construct an isobologram, but what we wanted to do was to look at the interaction of these two compounds using multiple endpoints, and I'll get into that a little bit here. Well, I'll go back.

Our quality -- Our hypothesis was that the interaction was going to be more than additive, because we thought that the interaction with the detoxification enzymes, that the presence of one or the other would inhibit the detoxification of the

other enzyme and, therefore, create more toxicity of the mixture than you would predict from looking at the two by themselves. So we predicted that their interaction would be supra-additive.

We used a dose additive model. Let me take a little bit of time with this, and I also have some examples graphically after this.

We spent some time reviewing the mixture literature, and it seemed to be that, if you wanted to look at compounds that had the same mechanism of toxicity, that are homergic -- is what they are called in the literature -- that you needed to employ the dose additive model.

To do this, what you do is you determine the ED_{50} for whatever endpoint you've decided to look at. In our case, we looked at two dose levels. We looked at what I would consider a very low dose level, which was the amount of the compound that would cause 50 percent inhibition of red blood cell acetylcholinesterase.

Then we looked also -- I did another group of experiments that looked at a higher dosage level, which was the amount of the compound that would cause 50 percent inhibition of brain acetylcholinesterase.

So we looked at it at two different dosage

levels, but in general what you do is you determine the ED_{50} for whatever your target endpoint is for both of the compounds. Then you take half that dose for each one of the compounds and put that in your mixture and look to see what the effect of the mixture is.

Your results can look like this, depending on what the interaction is. This is completely made-up data. That's why it looks so good. If you go up here at the very top, this is what your results would look like if you had an additive interaction.

So in this case, if we had -- This is the dose of chlorpyrifos that causes 50 percent inhibition in whatever tissue we're looking at. This is the dose of diazinon that causes 50 percent inhibition of that same tissue.

Then if you take half of those doses and mix them together and dose the animal, you should get -- If the interaction is additive, you should get 50 percent inhibition.

Now this is what it would look like if it was more than additive or supra-additive or potentiation or synergy, depending on who you read and how they define it. But this again would be the dose of chlorpyrifos that caused 50 percent inhibition. This would be the dose of diazinon, but together you

would get more inhibition if you used half of the dose. This down here, of course, is if you actually had antagonism or infra-additivity. There would be less inhibition in the groups.

So this was the model that we chose to use with these two compounds. The first thing you have to do is construct a very good dose response. You have to know what dose of chlorpyrifos or what dose of diazinon causes 50 percent inhibition in the brain or 50 percent inhibition in the red blood cells. That's what you've got here.

The x axis here is approximately the same for both of them, and you can see that there is a very different pattern here for the two compounds. The upper is chlorpyrifos. The filled symbols are brain, cholinesterase. The open symbols are red blood cell acetylcholinesterase.

You can see here that chlorpyrifos inhibits red blood cell acetylcholinesterase at lower doses than it does brain acetylcholinesterase. This is not news.

The same thing is true for diazinon. The various symbols, the various shapes are different experiments that we did. This is just to give you -- We had to repeat -- We had some data from other

studies. Then we did newer studies to combine with that data, and the same thing is true down here for diazinon.

This line right here is the 50 percent line. So if you look at diazinon here, you can see that at this dose of about 10 milligrams per kilogram, you get about 50 percent inhibition of the red blood cell acetylcholinesterase, but at a dose at about -- this is about 75 milligrams per kilogram, you get 50 percent inhibition of the brain acetylcholinesterase.

So there's a lot of work to be done up front with these type of studies, because you've got to have a really good dose response curve. Even if you had a really good one, it doesn't always turn out like you want it to.

So these are all the compounds by themselves. These are all acute dosing in corn oil in adult male rats. The animals were sacrificed at the time of peak effect, which conveniently for these two compounds is about the same time, which is about three to four hours after dosing.

Our endpoints: We had three different flavors of endpoints. We basically looked at cholinesterase inhibition, of course, in the brain, in the red blood cell, but we also did other

cholinesterase determinations in other tissues.

We did toxicokinetics. We're set up to look at the toxicokinetics of chlorpyrifos. So we looked at the distribution of chlorpyrifos in various tissues to answer the question of whether co-dosing with diazinon would cause a different distribution of chlorpyrifos and/or its metabolites in the liver and the brain.

Then in collaboration with Ginger Moser, she did behavioral assessment on these animals. So she ran an abbreviated functional observational battery on the animals.

So in the first experiment, which is the lower dose experiment, we used male -- adult hooded male rats, and our target endpoint was red blood cell inhibition -- red blood cell acetylcholinesterase inhibition of approximately 50 percent.

We determined the ED_{50} for chlorpyrifos to be approximately one milligram per kilogram, and that's the dose of chlorpyrifos that produces 50 percent inhibition in the red blood cell by itself, and diazinon to be ten milligrams per kilogram.

So what we did was we -- in our mixture group -- the mixture group consisted of .5 milligrams per kilogram of chlorpyrifos and 5 milligrams per

kilogram of diazinon. We had five dosage groups. We had a control that got nothing but vehicle.

We had the two groups by themselves. We had chlorpyrifos by itself. We had diazinon by itself. We had the mixture of the two at half, but then we also -- In order to compare the pharmacokinetics, we needed to have the chlorpyrifos at half the dose, at .5 milligrams per kilogram, because we needed to have that group to compare to the group, the mixture group, to see if the amount of chlorpyrifos and metabolites in those two groups were the same or had been altered.

You can't just assume that the amount of chlorpyrifos in the animal at one milligram per kilogram is going to be twice that as what you should see at five. So we needed -- I mean the .5. We needed the .5 to compare with the one. So that's a general outline.

So this is just to show you where we are in the dose response curve with regard to those doses.

So we had about one milligram per kilogram for chlorpyrifos and the mixture group. So that's the dose that we predicted would produce about 50 percent inhibition of acetylcholinesterase in red blood cells and about ten for the diazinon. So you can see where

we are here on the dose response curve.

So here are the results, same bars as I was showing you guys before, only this is real data. This is chlorpyrifos by itself at one milligram per kilogram. We didn't get exactly 50 percent inhibition, and diazinon at ten milligrams per kilogram, again a little bit higher than 50 percent. But you can see here that the mixture is right in between the two, which would indicate that we have an additive interaction at this low dose.

Same thing is true for plasma. We had a bit more inhibition by the two compounds in the plasma by themselves, but the mixture here produced a value that was in between these two, not higher or lower. We did not get any significant inhibition in the brain, which is what you would expect from our original dose response curves.

Now if you look at the toxicokinetic results, we're looking at -- At this low dose, we were unable to see any chlorpyrifos or any of its metabolites in the brain, but we did see trichlorpyridinol, which is a metabolite TCP of chlorpyrifos, in the liver.

If you look here, this is how much trichlorpyridinol is in the liver. This is an

nanograms per gram of tissue. This is how much trichlorpyridinol is in the liver of the animals that were dosed with one milligram per kilogram, and this is how much, and it's just about, conveniently, half. You couldn't have predicted that.

This is how much is in the liver at .5 milligrams per kilogram, and this bar here is how much trichlorpyridinol that was in the liver of the animals that received the mixture.

Now this is a different paradigm here. What you're looking at here is how closely these two bars resemble each other, because these animals got .5 milligrams per kilogram of chlorpyrifos, but they also got 5 milligrams per kilogram of diazinon. But you can see here that these bars aren't significantly different, and having that amount of diazinon on board did not change the distribution of the trichlorpyrydinol, presumably, and the chlorpyrifos in those animals.

There was no behavior to measure in those animals. They were not showing enough over-toxicity or behavioral alterations at those dosages for there to be any assessment in those animals. So I don't have any behavioral results for those animals.

Now in the second experiment -- This was

basically the same type of animal, Long-Evans hooded adult male rates. Our endpoint here is 50 percent inhibition of cholinesterase in the brain, not the red blood cell.

It was determined that the ED_{50} in this case was 20 milligrams per kilogram for the chlorpyrifos, which is 20 times higher than it was for the red blood cell, and 75 milligrams per kilogram for the diazinon, which is about 7.5 times higher than it was for the red blood cell.

Again, we had five dosage groups. We had a control. We had each one by itself. We had the mixture, and then we had animals that received chlorpyrifos at half the dose of it by itself so we could compare the distribution.

Again, our dose response curves here were at about ten for the chlorpyrifos for the brain acetylcholinesterase, and we're down here at 75 for the diazinon and for the brain acetylcholinesterase. Here are the results from that group of studies.

This is cholinesterase inhibition on the y axis. This is chlorpyrifos by itself. We did not have 50 percent inhibition in the brian. We only had about 90 percent -- 80 percent -- Sorry. We only had about 15-20 percent inhibition in the brain.

The diazinon, we did have about 40 percent inhibition in the brain, and the mixture was between the two. So, again, the interaction is additive. We've also looked at the retina. The chlorpyrifos produced about 60 percent inhibition in the retina of those same animals. Diazinon produced about 40 percent inhibition, and again the mixture group is in between them, indicating that the quality of interaction here is additive, even though we've used a much higher dose.

Diaphragm for peripheral tissue -- we just decided to look at this -- is basically the same thing. We had a lot less inhibition in the brain than we did in the diaphragm of the chlorpyrifos dosed animals than we did in the diazinon dosed animals all by themselves, but again the mixture was right in between.

So we don't see anything here in the biochemical results that would indicate that we've got anything more than just an additive interaction of these two, either at a low or a higher dose.

Now if you look at the toxicokinetics, we were able in the liver to see both chlorpyrifos, the parent compound, and trichlorpyridinol, the metabolite. Here again, you're looking at -- You're

comparing these two bars.

In the liver the level of trichlorpyridinol in the mixture animals was basically not any different than if you had given the same amount of chlorpyrifos by itself. These two are not significantly different statistically, but it does look like, if you did this maybe with 200 more animals, you might be able to get yourself a statistical difference between here. But all this is telling you is that maybe the presence of diazinon at this dose is inhibiting the conversion of the chlorpyrifos to its metabolites. But right now this is not significantly different and, therefore, we've got no reason to suspect anything more than additive.

We actually did see some trichlorpyridinol in the brains of these animals, and again these two bars are not significantly different. So the presence of diazinon did not change the distribution of trichlorpyridinol in the animals.

Here are the behavior results. We saw something a little bit different here. This is the motor activity in these animals, same method of presentation here. The chlorpyrifos animals alone saw about a 40 percent inhibition in motor activity. The diazinon animals you saw a slight inhibition in motor

activity.

The mixture group is basically between these two bars, and so you have an additive interaction. The mixture is where you would expect it if you gave half of the dose of these two bars.

Up here, this is the ataxia ratings that Ginger's group does. You can see here -- We've actually done this twice, because it was quite interesting the first time. This is the control animals. These are the chlorpyrifos by itself. These are the diazinon by itself.

In each case here you had one animal that showed a strange -- You've got a four or five point scale here. So this is slight ataxia in these animals. But in the mixture group we had four animals, five animals, that showed this effect, and each time we saw this.

I don't want to make anything too much out of this except that it was repeatable. so we've got here is an interaction at either a low or high dose that shows an additive interaction, the low dose being the dose that inhibited the brain -- I mean the red blood cell acetylcholinesterase, and the higher dose being the animals that had approximately 50 percent brain acetylcholinesterase inhibition.

In neither one did we see any type of -- any evidence that would lead us to believe that the interaction could be considered anything but additive except for some aspects of the behavioral testing.

These are the people that did all the work. Debbie Hunter does all the pharmacokinetic analysis. Lynn Lassiter and Renee Marshall help out in the lab with assessing the animals and also collecting tissues. Kathy McDaniel, Ginger Phillips and -- I mean Pam Phillips and Ginger Moser work with the behavioral assessment of the animals.

I'll be glad to take any questions, if anybody has got any questions. Nope? Okay.

(Applause.)

DR. NORRIS: Thank you, Stephanie. We'll take a quick 15 minute break, and we'll reconvene. Thank you.

(Whereupon, the foregoing matter went off the record at 10:49 a.m. and went back on the record at 11:14 a.m.)

DR. NORRIS: Okay. We keep getting off and on schedule. We'll try and stay closer to on schedule.

I'll introduce our next speaker who made it from Baltimore, thank goodness. In spite of the

weather? Dr. Will Boyes is the Chief of Neurophysiology and Toxicology Branch and the Acting Division Director for the Neurotoxicology Division right now at RTP in North Carolina.

I see folks still coming in. I did major introductions before you arrived to this morning's session, but I'll let you take it from here, Will. Thank you.

DR. BOYES: Well, thank you, Debbie, and I'm very happy to be here, especially given our episode on the plane this morning. We approached the Washington airport runway twice, and then the pilots said they couldn't see the runway. So we were going to Baltimore. I was looking at my watch wondering if I was going to make it or not, but they put us in cabs and we got here in good shape.

I haven't heard the morning's introductory talk. So if I repeat something, please forgive me. One more apology: I'm sorry I didn't put a biography in the thing. I didn't realize that it was going to be published like that, but I work in the same division as Stephanie and Ginger and, if you want to contact me, I'm in the EPA directory.

I am Acting Director for the Division at the moment while our division director is up here

learning what risk assessment is all about. So if I'm a little bit confused, I have a lot of excuses already.

Let's go ahead and get started now. People are sitting down. This is a set of studies that was done almost a decade ago, like what Ginger presented to you. At that time the EPA Superfund office was very interested in mixtures and funding research in the Office of Research and Development, and this is one of the projects that we ran with that funding.

This one was done with me as the project officer in collaboration with Chuck Rebert at the Stanford Research Institute. All of the data were collected out in Stanford at SRI.

We were interested in solvents. Before we designed the studies, though, we started looking at the literature. At that time, and I think it's probably still true today, the literature on mixtures in neurotoxicology is very sparse. There are very few studies published.

The ones that have been published have used largely in vitro preparations and, because of some of the complexities that I think you've already heard about, it's more practical to do it that way.

But it leaves a lot to be desired in terms of applicability to in vivo situations.

Those in vivo mixture studies that you find are largely, if they are not cancer studies, they are hepatotoxicity studies. There's very few in the nervous system, and there's even fewer with environmentally relevant compounds.

So we wanted to address some of those problems, but first why is that the case? It's a difficult thing to do mixture studies, and it's a difficult thing to study the nervous system.

The nervous system is a very heterogeneous structure. It has multiple target sized complex systems. You can have many outcomes. I think you saw this in Ginger's presentation. In some cases, there are very steep dose response curves, which can make the analysis difficult.

The difficulties with chemical mixture studies we're all hearing about today, that there are many chemicals, each -- If you look at complex mixtures, each complex site, each Superfund site, each source if you're talking about air pollution, might have a unique mixture, and how do you generalize from one complex mixture to another?

The designs, when you start looking at

multiple chemicals and multiple interactions, get very complex, and so do the analyses. So this is what you're faced with when you try and do these kinds of studies. But we forged ahead bravely.

We wanted to test the additivity hypothesis, because in the absence of other information, the EPA risk assessment assumes that compounds are additive.

We wanted to do this in whole animals with relevant compounds. So based on the interest from the Superfund program, we looked at a series of volatile organic solvents. They are not just of interest, of course, to Superfund. They are major components of EPA's portfolio for just about all the program offices.

With organic solvents in the nervous system, in humans the primary concern is usually cognitive function, but there has not been good models for cognitive function in animal studies. So we thought we would focus on a very definitive outcome that has been reported. It was actually discovered by Chuck Rebert in his collaborative work prior in the mid-eighties. That's damage to hearing, ototoxicity. I'll talk more about that in just a minute.

We wanted to use whole animals by

inhalation route, and to do this, though, we started with an abbreviated experimental design that's similar, in fact, to what Stephanie presented in the last talk.

This is a scanning electromicrograph from several rate cochlea that I stole from the dissertation of Ann Christian Johnson. These are stereocilia on three rows of hair cells. This is after just a couple of days of exposure to toluene. I think the exposure for 18 hours a day for three days to 1400 ppm high dose and extended exposures.

This is a couple of days later, and you can see that here they look normal and healthy, and here you can see the stereocilia beginning to break up, fall apart. Some of the cells are actually missing.

This is a couple of days after the exposure stopped. You can see there are whole areas where the hair cells, these outer hair cell, have disappeared, and there are other cells infiltrating to take their place a couple of weeks later, and there are whole regions of the cochlea that are devoid of outer hair cells. This will produce a profound hearing loss in those frequency bands in the cochlea.

This is from a review article by Gordon

Pryor. A lot of solvents were tested by Rebert and Pryor through the eighties and nineties, and this is from a review article that he published.

It's interesting. There is a very unusual and so far not well figured out structure activity relationship. The compounds that do produce hearing loss, and these are high dose, again, phenomena, but a lot of substituted benzenes, methylbenzene which is toluene, ethyl and propylmythoxybenzene and mixed xylenes. There are three xylene isomers, but when you test the isomers separately, only the para-isomer is ototoxic. The ortho and the meta- isomers are not.

Styrene and other substituted benzene is ototoxic, and then some -- and then monopyribenzene also, carbon disulfide, a little bit different solvent from these, and then some alkanes. Trichloroethylene is ototoxic and N-butanol. But then interestingly, these did not produce noticeable ototoxicity. Benzene itself, even though all the substituted benzenes -- a lot of substituted benzenes do, these substitutes don't, isopropyl, 1,2-dimethyl, 1,3, the xylene isomers.

Then comparing with trichloroethylene, dichloromethane, trichlorethane, tetrachloroethylene or perf doesn't. N-butanol is. 2-propanol is not,

and ethyl alcohol and N-hexane is not. N-hexane is the well known compound for producing peripheral neuropathy, like I imagine John O'Donoghue talked about, but I didn't hear him, because I was in Baltimore.

Okay. So I mentioned that we wanted to use an abbreviated experimental design. This sort of schematically illustrates what I'm talking about. It's very important when you look at chemical mixtures to do a dose response curve for your constituents of the mixture.

That's because, if you think of compound A here as just a typical dose response curve, if you do a mental experiment of combining a dose level like this of compound A with the same thing of compound A, a mixture of the compound A with compound A, and then that would be this dose plus this dose. So it would put you out here, and you could get a very profound effect by mixing compound A with itself, much greater than you would ever expect, and it's only because you've moved into a steep part of the dose response curve.

So if you don't understand the shape of the dose response curve, then you can fallaciously or spuriously conclude that you are getting a greater

than additive effect, when in fact you've just moved into a nonlinear part of the dose response curve.

So if you don't understand where you are on the dose response curve for your individual compounds, then it becomes almost impossible to interpret a mixture of different compounds.

This is the design that Stephanie talked about where she took a dose response curve for compound A and for compound B, took an equal effective dose of these, and then you add proportions of those doses together. If you add this ED_a to ED_b, then you would expect to get a very large effect. You can predict that based on the shape of the dose response curve.

We actually used these as our target dose in our experiments where we added fractions of the effective dose of compound A and fractions of the effective dose of compound B together, because we wanted a target on the steep part of the dose response curve so that we would be able to see changes that were both greater than or less than additivity.

So this is our basic strategy. This is a typical isobologram which you would take relative proportions of the compound A and compound B and add them together at different dose levels, so that you

have these different rays coming out, and then if they are additive, you would get a straight line between them at the point which produces an equal effect all along here. This is an additivity isobole.

If you get -- if it takes larger and larger doses of these compounds to produce the same effect, then that's considered to be less than additive or some people have used the term antagonistic. If it takes lower doses to produce the same effect, then that's termed greater than additivity or synergism.

We didn't have the ability to do that with these large animal studies. So what we've done is focused on the line of additivity, which you can see here, and take a 50/50 mixture of the doses that produced these effective doses, 75:25 and 25:75, and then 100/0 and 0/100 or vice versa, and add these -- use these dose combinations to test our additivity.

So if the compounds are, in fact, additive, then they should all produce the same effect. If they are greater than or less than additive, they should be statistically different from this linear extrapolation between the effective dose of A and the effective dose of B.

So that's the general strategy for our

experimental design.

This just sort of differentiates traditional from our simplified isoboles. Many of those levels for each compound when you apply the iso-effective concentration combinations, and it's practical in vitro, but it's very difficult to do in vivo unless you have very simple measures.

So we used our simple dose group at relative proportions, and then we're plotting them to see if the effects are different from the iso-effective. You can do this more practically with the kind of studies we want to do.

Nonadditivity will be demonstrated by whether or not the effects we see are different from that linear prediction.

This is kind of a repeat of just what I said. Dose response curve for each compound. Our mixtures are 0:100, 75:25, 50:50, 25:75 and 0:100 proportion of the iso-effective concentrations.

We picked five solvents from the list of positive ototoxic solvents: Styrene, trichloroethylene, toluene, mixed xylene isomers, and chlorobenzene. We did the same solvents on this side. When you do binary combinations of these, you come up with ten experiments that you can run.

We ran all ten of these combinations in this set of studies, first doing dose response curves for the individual solvents, picking the iso-effective levels, and then going back with a second study with five dose groups for each of these solvents.

We had about eight rats in each of these dose levels or -- I'm sorry, about 40 animals plus a clean air control for about 50 animals, 48 animals, for each of these ten experiments. So you can see, even with these simplified designs, when you look at multiple chemicals, it becomes a very large endeavor.

The animals were inhalation exposed five days, eight hours per day, Monday through Friday. It's enough to produce permanent ototoxicity. We waited about ten days before we started testing their hearing.

This is how we tested hearing. We didn't dissect out the cochlea and do scanning electromicrographs like you saw in that beautiful slide of Ann Christian Johnson's. We have a much more efficient way to do this, and this is the electrophysiological method called the brain stem auditory evoke potential or some people refer to it as the auditory brain stem response, ABR.

To do this, you anesthetize the animal so

that it doesn't move. That's the main thing you want. You can put needle electrodes under the scalp, maintain body temperature so that's not a concern, and then stimulate the ears with tone pips.

You can make the sound loud or you can change the frequency from low to high. Now what we report is a response that's the mass discharge of cells in the auditory system. There's a lot of work that's gone into the generators of this potential, and we know that the first peak we measure is generated -- There's a little response in the cochlea. The first major peak is in the auditory nerve.

Here you can see the eighth nerve or the acoustic nerve. Then the next peaks are generated along the line of the ascending auditory pathway, and the neural generators for each of these are pretty well identified, the cochlea nucleus, superior auditory complex, lateral aniscus, medial tinnicular.

So we can follow the ascending auditory signal as it goes up the auditory pathway. This basically says what I just said. Anesthetize the rats. Stimulate varying and loudness in pitch, recording the electrical activity.

Then we needed a simple measure. There are a lot of measures you can take off of this, but

because of our complex design, we wanted a single dependent variable. What we settled on was the integrated amplitude of the response between 55 and 85 decibels. I'll show you this in just a minute.

This gives us a very nice and regular measure that's sort of a global indicator of the neural function in the auditory system. Let me show you the next slide, and that will become clear.

These are examples from groups of animals averaged together at 25 to 95 decibels sound level. In the control animals you can see that it starts out at 25 decibels with a very small response, and it grows in amplitude -- this is voltage on this scale, and this is time on this scale -- and becomes earlier in latency as the response -- as the stimulus gets louder.

P1 is the peak generator in the auditory nerve, and you can see that it follows up and down with a nice relationship to intensity. What we did for the dependent measure was to take between here and here, rectify it so that it was all positive, and then do an area under the curve. It gives us just a summary measure for the function of the auditory system.

Chuck decided to it. I think it was

between 25 and 85 decibels. So he summed all of these up. I think he left off 95 decibels, because of the possibility of auditory recruitment, which happens in some animals with hearing loss.

What's interesting here is the effect of styrene. Here you can see after 2000 PPM styrene for five days many things. First of all, the amplitudes are much smaller, but more importantly, the threshold for eliciting response is tremendously changed.

What you see, about 25 decibels in the control, you're seeing at around 65 decibels in the exposed animals. This is a profound hearing loss, about a 40db loss of hearing. So that shows you the kind of an effect that we're talking about.

Now I didn't bring all the data. I wasn't sure how much time it would take me to go through that part of it. But this just samples dose response curves for the individual compounds alone. This is chlorobenzene, and this is toluene. This is kind of the best and the worse.

The toluene looked very nice. We had a nice linear response curve. Chlorobenzene -- we had a little trouble with this particular compound, not the others, in that at high doses the animals lost a lot of weight. So we couldn't go much higher, and we

had a couple of uneffective dose levels. So we didn't have a lot of room on the chlorobenzene dose response curve to work out things.

Let me show you the next slide now. This is sort of -- We ran ten experiments. One of them, we had to throw out because the solvent generation apparatus basically failed, and the animals got exposed to what they weren't supposed to. But the other nine all gave us nice data, and we felt very confident about that they were exposed to what they were supposed to be.

We did have some trouble with some of the experiments, and I'll talk about that in a minute.

This is what we expected to see. This is trichloroethylene and toluene. The control animals had a very large integrative amplitude, about 200. The trichloroethylene alone produced about a half a maximal effect. This was exactly what we were shooting for, and the toluene produced about the same thing.

You can see that the 25:75, 50:50, and 75:25 combinations produced effects that were equivalent to those of the compounds alone. This is exactly what we expected to see with additivity, and this is the equivalent to the graphs that Stephanie

was showing in the last talk.

The problem that we had was that we saw drift in the dose response curves for the individual compounds alone. Here, xylene produced about what we wanted to by itself, but here trichloroethylene at 2800 PPM is less effective than it was here at 2600 PPM. But still, the combinations between these two produced a linear relationship.

Dave Sensgaard did a lot of statistical analysis with us, and demonstrated that, even though our individual compounds were not iso-effective, that the linear relationship between them was indicative of an additive effect.

Here you can see a similar effect. Here the xylene was not as effective as we thought, but again in combination with chlorobenzene, it was a linear relationship between the effects of the two compounds.

Similar here, this is chlorobenzene at 2000. This was chlorobenzene at 2400. So again you can see there's quite a drift in the different attempts to ascertain the dose response curve for the individual compounds. But the simultaneously run mixtures here with toluene showed again an additive interaction.

This is four of the ten experiments, but without showing you all the data, what we found was essentially in all the experiments there was a linear relationship between the different combinations. so we did not detect any nonadditive interactions.

Now let me go back and make one more point from that slide. One of the things that I think is our lessons learned is that, obviously, you need to have -- It would be nice to have stable dose response curves to do this kind of work.

Another thing is that it's not clear to me how big a difference from additivity we would have been able to detect. So it would have been nice to have compounds that we knew were nonadditive to see if our design was sensitive enough to detect them.

We did some mathematical calculations, but it would have been nice to have some positive control compounds.

So a couple of conclusions: For the ototoxic solvents we saw no deviations from additivity. This suggests that the compounds are substitutable for each other, that they cause the same thing. And given that they are all organic solvents and they produce a fairly similar hearing loss, one possibility is that this suggests a common mechanism

or mode of action. It doesn't prove it, but that is a possibility.

Lessons for the experimental design: I think that our simplified analysis of the isobolograms was effective at allowing us to test binary mixtures. We had, as I pointed out, problems with the instability of our dose response curves for the individual compounds and, as I mentioned before, it would have been nice to have a positive control to differentiate between our ability to detect additivity and nonadditivity.

We weren't able to follow these studies beyond this. But one of the things that I would have liked to have been able to do was to look at noise. Noise is a very common cause for hearing loss in occupational settings, although it's not currently something EPA is concerned about. But other people have looked at workers exposed to solvents in the presence of noise.

The conclusion has been that it does tend to cause a greater than expected hearing loss for noise when you are exposed to solvents. So I think that's something that is a very interesting outcome from this work.

I'd be happy to take any questions. Thank

you.

(Applause.)

QUESTION: Why do you think the baseline drifted?

DR. BOYES: I don't have the answer to that. We did take simultaneous blood levels from all of the animals during all the exposures, and they were fairly constant. It doesn't look like it was exposure of the animals to the solvent or absorption into the blood.

It was something in the different batches of animals or the different time of year or whatever it was when we ran the studies that the animals differed in their sensitivity to the solvent. I wish I knew that, because I don't.

DR. NORRIS: Thank you, Will. Our next speaker, Dr. Greg Christoph.

DR. CHRISTOPH: Thank you, Deborah, and thanks to the organizers for inviting me here to talk, as I'm just preparing to leave the industry. It was mentioned earlier that I am retiring tomorrow, and actually I'm not going to retire. I'm going to -- Well, I am retiring, but I'm going to start a different career with a different kind of industry and a different kind of regulatory agency.

So this is my opportunity just to say exactly what I think. Well, indeed, I think I've always said exactly what I thought. At least I hope I have, and perhaps that's one reason why I'm retiring as a director instead of a senior vice president. But I do work for the DuPont Company as of today, and I'm sure all of you are aware that DuPont is a very, very large chemical company and makes a number of the kinds of chemicals we've been talking about here today.

In particular, since I am going to be talking about pesticides, DuPont does have an agricultural chemicals business, and they do make lots of different kinds of pesticides. In particular, they make an organophosphate cholinesterase inhibitor. They make carbamate cholinesterase inhibitors. They make a pyrethroid sodium channel opener that is an insecticide, and soon they will be producing a pyraziline insecticide which is a new and different kind of mechanism of action which, nonetheless, affects sodium channels.

So we make a lot of different chemicals that are sort of, by definition, neurotoxic or have neurotoxic potential in animals anyway because of their known mechanism of action.

I'm going to be talking primarily about

theory, very little data here, in fact, no data that I've produced myself, some tiny amount of data from the literature; but it's really the ideas about cumulative risk assessment that I would like to talk to you about, particularly in the context of the Food Quality Protection Act and how cumulative risk assessment is envisioned in the context of the Food Quality Protection Act.

Basically, the steps in cumulative risk assessment are fundamentally no different, I think, from those in any kind of a risk assessment analysis. There's four fundamental categories of activity.

First is hazard identification. There's dose response characterization, exposure estimations, and finally the risk assessment itself where all that information is pulled together. The nature of the events or the specific events within each of those categories are a little bit different, however, for the cumulative assessment process.

First of all, the hazard identification step -- really, the analog of that in cumulative risk assessment has to do with deciding what the common mechanism of action is for the chemicals. It is generally decided that something like inhibition of the cholinesterase enzyme is a common mode of action,

and that itself poses a hazard.

So that kind of hazard determination, while there's a lot of discussion about that and what needs to do into that, is typically -- When that determination about the common mechanism of action or common mode of action is made, that's essentially the hazard identification step associated with the group of chemicals.

The dose response characterization step really becomes an issue in deciding what the identical critical endpoint in the same species for all compounds is, and those are kind of ideal kind of statements. It would be very, very -- It would be best is, for all the compounds in the risk cup, if the same endpoint were used. So red cell, cholinesterase inhibition in female rats, for example, is common critical endpoint.

That's not always going to be possible for certain groups of compounds, for pyrethroids, for example. One may have a more sensitive endpoint which might be muscle fasciculations in one case, and it might be a mild limb tremor in another. You have to decide whether those kinds of endpoints are similar enough to be counted as the same thing and do the modeling and the risk evaluation activities off those

kinds of qualitatively similar or probably mechanistically related sort of data points.

Dose normalization is something that happens, is absolutely necessary to do within cumulative risk assessment, and it's blue here because that's what I'm going to spend most of my time talking about today or at least one of the things I'm going to spend most of my time talking about.

Exposure estimation is a huge area. In fact, all these things are huge areas and require a lot of discussion, and I'm only going to talk about a few of them today. But basically, the way exposure estimation works is some kind of a Monte Carlo routine which assembles data associated with the foods people really eat, the kinds of pesticides that are used on those foods, the kinds of quantities of pesticides that potentially occur on those foods, and the kinds of other activities that people get involved in, because the cumulative risk assessment process involves both an aggregate and a cumulative risk analysis, all combined into one thing.

Then, of course, there is the addition of the normalized dose units, and I'm going to spend some time talking about that process.

There's some knowledge of how we are going

to apply uncertainty factors to the analysis. I'm sure everyone is familiar with the way uncertainty factors are used in risk analysis or risk assessment for a single compound. The way those uncertainty factors are used, I think, is a little bit different, and certainly it's more complicated in the context of a group of compounds that share a mechanism of toxicity.

I'm going to spend some time talking about the time frame, the appropriate time frame over which exposures ought to be considered for addition, and certainly, the kinetics of the individual compounds in the risk cup matter in that context.

Finally, the risk assessment itself in which all the data are assembled together and some kind of an outcome is handed over to a risk manager. Now my personal opinion is that the kind of information that the risk manager should get is the frequency of their expected or potential -- potentially expected frequency of adverse effects in the population.

So many people out of a large number, like 265 million, might be expected to experience an adverse effect due to the presence of this combination of pesticides used in the marketplace.

While I think that's kind of where we should be driving, the analysis, as I understand where it's going, is a little bit different than that in that it basically is a more sort of a point analysis. That is, well, this group of pesticides has an unacceptable margin of exposure. So we have to do something about that.

I think we can make a more intelligent decision if we knew something about the actual frequency of adverse effects and are actually worried about calculating that kind of value.

As I said, all these are very long conversations in themselves, and I'm simply going to focus on the ones highlighted in blue here.

First of all, dose normalization: Essentially, this is the theory underlying justifying how we are going to be adding doses together. So it's worthwhile to talk about that theory.

Essentially, on the top figure there on the right we have two pesticides, and let's say they are cholinesterase inhibitors and the experiments are similar, say 90 day rat studies in which we have sampled red cell cholinesterase inhibition, and we have a dependent variable there listed on the abscissa, and -- no, let's see, ordinate. The

dependent variable on the ordinate is -- let's say that's cholinesterase inhibition. This is clearly totally invented numbers that fit a very pure mathematical function, in this case the logistic function, which is fit to them.

The two compounds have different potency in this regard. The compound on the left is ten times more potent than the compound on the right. So how are we going to add exposures of these things together?

Well, we have to normalize the doses -- or we have to normalize them somehow to make them appear as though they had comparable potency. So there is an adjustment that's done.

The one that's shown here on the lower panel, the left figure in the lower panel, shows what happens if we compute the ED_{50} for the compound on the left and divide all the doses used in the actual experiment by that ED_{50}. We can express the dose for that chemical in ED_{50} units.

So, two, we take the ED_{50} of the compound on the right and divide all the doses in that experiment by the ED_{50}, and then we express the doses then in ED_{50} units. So then we can pull those two things together, and in the lower lefthand figure

there you see what happened.

The data points are probably maybe, unfortunately, too small to see, but the four data points for the two chemicals making eight data points altogether now appear on a single merged superimposed function, when the doses are expressed in common units, in this case common ED_{50} units. The dose response functions are perfectly superimposable.

Indeed, this is an assumption of the analysis, that the dose response curves are parallel. parallel is really the wrong word, because they are not linear functions. They are sigmoidal functions or curvilinear functions, and in this case the best way to -- more accurate way to describe it is the dose response functions share the same slope parameter, but I think you know what I mean. The dose response functions are parallel.

Under those conditions, expressing the dose according to a common effect level, in this case ED_{50}, has the effect of superimposing the dose response curves.

Now in this case, what happens is that the -- one can take any number of ED_{50} units of any of the chemicals in the mixture and add them to another one, and that result, that sum, will lead to a predictable

effect. So for example, one could take .1 ED_{50} units of chemical A and .1 ED_{50} units of chemical B and .1 plus .1 is .2, and we can simply march up the dose response function in the lower lefthand panel and say, okay, where does .2 ED_{50} dose units bring us in terms of the predicted effect.

Perhaps it would be better to deal with a slightly larger set of numbers like, let's say, we had -- If we had .5 ED_{50} units of compound A plus .5 ED_{50} units of compound B, the total is 1 ED_{50} unit. We would expect 50 percent inhibition in that case.

This is true regardless of the number of the chemicals in a risk cup. If there are ten or 34 chemicals in the risk cup, if we're adding up and they all have the same slope and all have the same parallel dose response functions, when we normalize the doses according to a common -- the dose associated with a common effect level, that has the effect of superimposing the dose response curves and making it justifiable that we're adding doses to lead to a common effect.

This is fundamentally what we're doing in any kind of a dose addition cumulative risk analysis. We don't always say we're marching up the dose response curve like that, because very often we are

looking for some kind of a point threshold, whether you exceed it or not, that might be important. But essentially what we're doing is adding doses and tracking up the dose response curve.

There is a tendency to say, well, we can't really express doses on ED_{50} because the data aren't good enough to actually get our handle on a good estimate of the ED_{50} for all the compounds in the risk cup. That's probably true. Probably the actual regulatory data that you have in your hands that are submitted to you by companies probably is not always good enough to produce an accurate estimate of the ED_{50} or an ED_{10}, for that matter.

So it is tempting and probably necessary as a practical matter to, instead of using a common effect level like ED_{10} or ED_{50} and to normalize dose units, rather instead to use the no effect level of the two compounds. That's shown here.

So the experiment here has a no effect level of .1 mgs per kilogram. The compound on the right has a no effect level of, looks like, 3 mgs per kilogram, and then we could normalize the doses by dividing each of the doses in the experiment by the no effect level dose. Now we're expressing doses in terms of no effect units, NOEL units, basically.

So one NOEL unit in both cases is a no effect level for both compounds. Note that what happens, because of the arbitrariness of how the experiments are conducted and dose selection and all the different kinds of rationales that go into exactly how the experiment is conducted and statistics and everything else, when such a transformation of dose is made, the dose response curves are no longer -- are not superimposable.

In essence, when we're doing that, now when we're adding up NOEL units of compound A and compound B, we don't really know which line to track up to know what the predicted level is going to be, and that's simply a consequence of the fact that the functions are not superimposable like they are on the left.

So for this reason, it's technically inappropriate to use something like a NOEL, but obviously, until we get better data to the agency, I think we are probably going to be forced as a practical matter to do that.

Now on the positive side of doing that, probably the errors are not going to be too great, because where we are located down here on the low end of the dose response curve is -- things are going to

be reasonably similar to each other most -- well, we're pretty sure that doing such an action certainly would not put the public at risk. It's simply not totally scientifically appropriate to do it.

There are other issues pertaining to non-parallel dose response functions. I was in a hallway conversation earlier: What do you do in the case where the dose response functions are not parallel?

It's an interesting problem, because -- Well, technically, we really shouldn't be adding the doses together in that case. As a practical resolution to that, instead of comparing -- or normalizing doses around ED_{50} for example, what one might do there would be to normalize doses around a lower effect level such as an ED_{10}, and now express the effects in terms of ED_{10} dose units.

The reason for doing that is because, when non-parallel dose response functions are normalized around an ED_{50}, essentially they become superimposed at the inflection point of the sigmoidal dose response function around the ED_{50} point. So one could imagine one compound having that slope and then the normalized function intersecting with that, but following that sort of trajectory.

What that does is displace things on the

low end a lot more than if one were to normalize around an ED_{10} in which case the compounds of dissimilar slopes are now fairly close together on the low end where we are actually going to be adding things up where things really matter, and are more disparate at the higher dose levels where people aren't really exposed to those kinds of levels anyway. If we get up to 50-60 percent inhibition, we know we are close to that in human beings. So that would be how to handle that problem.

Well, in kind of the real world, how does this work, this idea of normalizing dose response functions to something like an ED_{50}? Here we have seven organophosphate cholinesterase inhibitors. I just labeled them A,B,C, and so on here. But in fact, you know, they are things like chlorpyrifos and your basic list of typically used organophosphates.

These are dose response functions for red blood cell cholinesterase inhibition, all taken from 90-day rat dietary studies. You see here that the seven compounds have -- They vary almost a factor of 100 in potency. In general, the dose response functions are fairly similar in slope.

They are not so markedly different than one would say those are really different. Certainly,

they are not statistically different, although interestingly, there are two groups of disparate -- two groups of different slopes here, but they are not that different. Actually, I think it's the ones with the white data points that mathematically end up having slightly less steep slopes than the others.

Let's go through that exercise of now determining the ED_{50} for each of these compounds and expressing the doses of that compound in ED_{50} units and going on through that entire process for all seven compounds, and then assembling all the data together in the same graph and seeing what happens.

That's what's going on in panel B down here in which we have percent inhibition of cholinesterase as a function of dose expressed in ED_{50} units. You can see that the data points from the seven different experiments all pretty much fit reasonably nicely a logistic curve function, and here are the parameters associated with that logistic function.

One can compute a 95 percent confidence -- set of 95 percent confidence intervals associated with that logistic function, and from that 95 percent confidence interval one can determine that an ED_{20}, a dose that causes 20 percent inhibition of red cell

cholinesterase, is 0.34 ED_{50} units, and that's the intersection of the upper bound of the 95 percent confidence limit with the 20 percent inhibition level.

So regardless of the OP that we have here that differ by a factor of 100, in general 0.34 ED_{50} units, whatever the ED_{50} of the particular compound is, is going to produce about 20 percent inhibition.

If one does not like ED_{20} as some kind of a point of departure for risk analysis, you might like ED_5 or ED_{10} or some other number. What I'm just showing you is simply a matter of whatever number you like. If you like ED_{10}, you can determine what the intersection of the upper bound of the 95 percent confidence limit with the ED_{10} is, and determine that that is 0.2 ED_{50} units.

Once you have that number, then one can sort of very readily move forward with a dose addition process. So when you know that an apple has X ED_{50} units on it, then you could add that to Y ED_{50} units of a pear -- of a pesticide that's on a pear, a different pesticide that's on another piece of food, for example.

So I just mentioned the process or the POD -- that is, the point of departure from the experimental data. I simply want to define that for

you. That's the number from which all further analysis is going to occur, and whether that point of departure is a NOEL or an ED_{10} or an ED_{20}, we're simply going to call that a point of departure to have some language that's useful in the analysis.

We need to have some kind of a measure or assumed or assigned values associated with the pesticides that people consume. So we're going to have to know the amount of pesticide residues on foods or be able to estimate that in some way.

We need to know how much food is consumed by an individual and the kinds of foods that they eat, and when they eat it; and we certainly need to know their body weight.

Basically, these kinds of things occur in the context of a Monte Carlo algorithm that assigns them based on distributions of their occurrence in the real world. Then we're going to go through a process of computing the normalized units of exposure for each of the compounds.

So a person might eat an apple that has 12 micrograms per kilogram of chlorpyrifos on it, and we're going to have to convert that amount of chlorpyrifos to normalized exposure units and then determine what the dose in normalized exposure units

for that compound on that apple are for that individual.

This whole process can -- You know, now we have to express the cumulative exposure, and there are a number of different ways of getting there. The mathematical -- Well, the arithmetic -- It's not mathematics, really; it's arithmetic -- by which one expresses these cumulative exposures can look really different, depending on which kind of metric one chooses.

One way to do it is to calculate a margin of exposure. Another way is to calculate the milligram per kilogram equivalents of a particular index chemical, and there's a lot of interest in saying that chlorpyrifos, for example, is an index chemical. It's certainly a widely used chemical that's been fairly well studied, and we can express all other organophosphate cholinesterase inhibitors in the same -- as milligram per kilogram chlorpyrifos equivalents.

Alternately, we can say how that exposure fills up the risk cup. So we have a risk cup which is allowed to hold 100 percent of one's permitted allotment of organophosphate cholinesterase inhibitor, and as one eats more foods that contain more different

kinds of pesticides, more different kinds of OPs, we're filling up that risk cup.

Arithmetically, the calculations look like quite different. Mathematically, they are absolutely identical. So it doesn't really matter if we're talking margin of exposure or relative potency factors in milligram per kilogram equivalents or we're filling up a certain percentage of the risk cup. The numbers are quantitatively exactly the same, because there is a mathematical identity underlying all those computations.

The reason that the answers might be different is if one is not careful about handling the uncertainty factors that figure into the equation. If one handles the uncertainty factors in the same way, all the values will come out exactly the same. They are all directly translatable to each other in a precise quantitative way.

Well, let's go through a hypothetical exposure scenario here, just to show you how this might work in the context of a margin of exposure kind of analysis, and I've just made up these numbers. These numbers have no meaning at all.

First of all, we are going to define the margin of exposure as the rat POD, the normalized rat

POD, divided by the human exposure, the normalized human exposure, sort of expressed in the same kind of unit.

So a rat POD might be one -- or .1 ED_{50} units. Actually, I said it was .34 ED_{50} units before when I was going through my exercise, and that would be a .34 ED_{50} units provides 20 percent red cell cholinesterase inhibition in the rat.

Well, what is the actual human exposure on that apple, for example? What is the normalized human exposure? What is the ratio of those two? That's the margin of exposure. Clearly, the higher that number, the larger the difference is between what it takes to affect a rat and what the human receives.

We can calculate an MOE for the entire chemical, and that's simply done by adding the reciprocal MOEs and taking the reciprocal of that. In this particular example, I've had a person eating -- On a given day, I had them eat one kiwi fruit, 12 grapes, four slices of bread, two slices of pizza, ten ounces of cornflakes, 16 ounces of green salad, 1 ounces of spaghetti with tomato sauce, and 14 apples.

Then we have chemical A, B, C and D. So if we want to determine what the MOE for a particular chemical is, we compute what the MOE is for the kiwi

fruit, what the MOE is for the apples, what the MOE is and so on. Then we can figure out what the total MOE for the chemical is.

In this case in my hypothetical example here, the person has consumed -- has a 386 margin of exposure. That would -- Whether that's safe or not or harmful or not depends on where the uncertainty factors are associated with that and whether there will be some kind of a threshold above which or below which we would no longer regard that exposure as safe.

In general, at this point in time and probably it will change soon, is changing as we speak, right now an acceptable margin of exposure might be regarded as 100. That incorporates two uncertainty factors, animal to human, uncertainty associated with the animal to human extrapolation, and a factor of ten for variation in sensitivity within the human population.

So ten times ten is 100. That number might go up if, one has more uncertainties about things like unstudied effects in developing animals, but for now let's just say it's 100. So 386 is a larger value than 100. So one would say, well, the MOE for that chemical, that particular chemical A, is okay.

So we go through the process and simply now look at the exposures associated with these different foods which have chemical B, C, D and E and so on, and indeed this would actually extend quite a bit further. There are 34 OPs registered for use in the United States, and one could imagine -- You know, one has to deconstruct things like pizza. Well, pizza has tomato sauce. It might have green peppers and onions on it. It has dough, flour, which is grain which is -- pesticides are used on that.

A slice of pizza might have five or six different pesticides on it altogether. Spaghetti is made with flour as well. It's got tomatoes in it. So a particular food -- and the Monte Carlo algorithm does all this deconstruction of foods based on standardized data that comes out of the -- I think it's USDA.

So once one figures out how the values ought to be assigned -- and here the exposures are expressed in MOE units -- one gets an MOE for each chemical. Then one has to determine what the total cumulative margin of exposure is, and that's a similar kind of equation except now we're adding up the MOE values or adding up the reciprocals of the MOEs to get the cumulative margin of exposure.

In this particular example, the cumulative MOE is 54. That is less than 100 or we've filled up 184 percent of the risk cup. This particular combination of events would probably be regarded as unsafe.

That is really just one iterative step in the Monte Carlo routine that assigns pesticides and pesticide levels to foods that are also assigned to individuals. That iterative process is executed 10,000 times or more, and from that, the total MOE, one obtains then the distribution of MOE values.

Here I've simply invented a distribution of MOE values to show you how this might work, in which case we have the average, in this case, total MOE is on the order of 900 or so. So the average person is probably pretty safe. But there is a small group of people down here -- in fact, it's .82 percent of the population -- that might be expected to have an MOE less than 100.

The risk manager getting this information would then want to decide whether something needs to be done about this. He can either look at the events that went into that .82 percent of the population, what was it that they were doing? Were they eating apples? Was it pizza that did it? You need to

maintain the linkages in the Monte Carlo routine to get back to the specific chemical events that led one there.

So that's kind of how the process works now, as we understand it. I want to take a short aside here and mention that the -- about getting to the frequency of the population that's affected.

This is right now what would go to the risk manager. .82 percent of the population has an MOE of less than 100; what do we need to do about that? I think it might be worthwhile if we take that extra step and try and figure out what the frequency of the affected individuals in the population is.

To know that, it's not .82 percent of the population that's affected. I mean, that's a lot of people in a country the size of the United States. Let's remember that the underlying assumptions that go into the risk factor analysis -- or the uncertainty factor analysis in this -- One of them, for example, there's a tenfold variation in human sensitivity. How frequent is that sensitive person in the population?

The bit of thinking I've done about that problem, it's actually a pretty rare individual. It's certainly as rare as one in 1,000 persons is going to be ten times more sensitive than the average person.

It's probably more like one in a million people is going to be ten times more sensitive than the average person. That's based on an analysis of a lot of pharmacological data, because the drug companies have a lot of information on that.

So given your standard drug, what is the variation introduced -- or in human sensitivity to drugs? Those numbers are on the order of a factor of ten has a probability of something like one in a million or certainly more than one in 1,000 or less than one in 1,000, probably something more like one in a million.

So when we're looking at .82 percent of the population, that's .82 percent of the population that's one in 1,000 or one in a million in itself. So it's a very small fraction of a very small fraction. We're not talking about millions of people being affected by this .82 percent. It's quite a bit less than that.

Okay. In a few minutes I'll say something about the kinetics and trying to get to an analysis of the time frame that one needs to go through in this process.

If our data were based on that 90-day study -- remember, that's a repeated dose, 90-day

study; the animals receive that dose every day for 90 days, and we determine what the POD or the NOEL was in that experiment.

What I just went through with the Monte Carlo analysis really involved just a single day -- Okay? -- analyzing the dietary life in a single day of this hypothetical individual and generating a normalized distribution in that single day. We ended up concluding that .82 percent of the population in the hypothetical example might have had a problem.

Okay. Does that .82 percent of the population really have a problem, because in fact that's just happening on one day? Remember, that individual was eating 12 apples or 14 apples, was eating a ton of spaghetti, very, very large salad, and I guess, just sort of apply some common sense here. How many days in a row are you going to eat 14 apples? I submit, not many.

So what we really need to do is to start thinking about how those daily exposures really occur in time. If one is at the 99.9 percentile of the exposure distribution on one day because they eat 14 apples, is it likely they are going to be there on the next day and the next day and the next day; because, let's face it, in the context of a long acting set of

compounds like organophosphates what's going to happen is that it's the daily exposure that accumulates over time that builds up to that sustained daily exposure that causes the cholinesterase inhibition to build up to a particular level.

I've modeled this process extensively. The modeling -- The details of the modeling are important if you were to accept my quantitative conclusions, and I really don't have time to talk about sort of the detailed model here. But again just sort of -- It's the qualitative conclusion which is the take-home message here.

So I think what we really need to do here is to just ask, well, what is the accumulated cholinesterase inhibition? Here we have percent inhibition as a function of time in hours. Zero to 1500 hours is two months.

So a compound like chlorpyrifos which has an acute half-life of about 175 hours, one consumes a little bit one day one. Then that produces some cholinesterase inhibition which decays with a half-life of 175 hours. On day two, another amount of chlorpyrifos is added to the diet. That is going to increment the amount of residual cholinesterase inhibition that occurred from the day before, and so

on and so on and so on.

Clearly, you can model this process. That's what I've done here to generate this kind of function. This basically describes the progression of cholinesterase inhibition in a rat administered chlorpyrifos every day over a period of two months.

One can see that it takes about -- actually, about a month to get to 95 percent of the steady state. So that's an animal that's eating one POD unit -- That rat is eating one POD unit to get -- and POD is defined as the amount it takes to get to 20 percent inhibition in a subchronic study. That rat -- By eating that one POD unit every day for two months, in fact, that's what it takes to build up to that level.

Now what if the person were to eat -- not person -- that rat were to eat one POD unit on a single day, never had any on any other day? Well, that's shown right here. In fact, what that does is produce a very small two percent change in the dependent variable.

Basically, the way the risk analysis is set up now, as I understand it, we're basically treating that two percent effect as if it's a 20 percent effect, because we are only focusing on one

day of exposure. We need to focus on the multiple day to day to day exposure scenario, because that's the experiment that's driving our analysis, not a single day of exposure.

So we need to open up that process. In fact, if you compute what it would take for a single day exposure to get to 20 percent cholinesterase inhibition, which is kind of where our hypothetical point of departure is for this experiment, it takes four and a half POD units.

So one POD unit might be the amount of pesticide residue that's, say, on 20 apples. If you are never exposed to chlorpyrifos, you could probably eat 4.5 times 20, some 90 apples, on a particular day, and you would still be below the threshold that might be regarded as adverse.

So it's simply by examining the kinetics and modeling that process, one can get at least a qualitative flavor that we need to focus on, land on more than a single day of exposure.

Clearly, the individual kinetic properties of the compounds in the risk cup make a difference. It takes a different amount of time to get to the steady state level. Here we have three different OPs. A compound like azinfosmethyl has a half-life of 35

hours. Chlorpyrifos is 175. Chlorthoxifos is 125 hours, and you can see that these different kinetics mean that steady state is established after different periods of time.

For chlorpyrifos it takes about 30 days. Other compounds with quicker half-lives will get there quicker. In a cumulative analysis the time frame that we need to sort out is equal to that which has the longest -- well, the compound with the longest half-life in the analysis.

So probably what we would be needing to do in the case of organophosphates is to focus on 30 days of dietary -- daily dietary consumption and try and figure out what the process over 30 days looks like.

What would happen there? What's the right metric. Once we've determined that the right time frame for OPs is something on the order of 30 days, what kind of metric do we do within that 30 days? Do we choose the very, very highest value that occurs in 30 days or do we choose the mean value?

Well, the mean value is certainly a lot closer to the one we should be using rather than the P. Here is an example of that. I've gone ahead and now computed what the cholinesterase inhibition, the expected cholinesterase inhibition would be over time.

Here we have cholinesterase inhibition over two months of continuous administration or daily administration anyway.

Here on the graph on the right we have the exposure units that go into this. Again, I've just programmed my computer to make up values to introduce some variation in them. The average over this 30 day period is about .39 POD units. So that's like .39 of a NOEL is administered to this rat here.

Now under those conditions where one receives kind of an average of .39, you see that we build up to a steady state which looks like about three percent inhibition here under those conditions, with even a spike of activity I forced in here of two and half POD units. That would be like going from the 20 apples to 50 apples. So eating 50 apples on a day, one still does not get anywhere close to the level of cholinesterase inhibition that would be regarded as adverse.

Now turning this kind of analysis to the more cumulative case, in this case we have three different OPs, A, B, and C. Here is the exposure scenario. Everything is randomized, so different amounts of compound A on every day of the two months, different amounts of compound C on every day of the

two months, and different amounts of compound B in every day of the two months.

During this two month exercise of daily diet, you can see here -- Let's just focus on the last month of exposure here. We have one, two, three, four, five, six, seven, eight, nine -- nine out of the 30 days in which this animal in this -- While this is modeled after animals, one could think about this as the one in 1,000 person that's more sensitive than the average person and also assuming that the average person is ten times more sensitive than the average rat. Under those kind of conditions, you can think about these data as being a human exposure scenario.

So nine out of these last 30 days contain an exposure which is -- in the present context, would be regarded as excessive, but the degree of cumulative inhibition is just getting to the point where it's 19 percent inhibition. It does not exceed our threshold of 20 percent.

So even though we've busted the line on exposure, the process of actually modeling the effect according to using more of an average like .8 -- the average of these values are less; 30 days is .8 POD units, which brings us right there.

So, clearly, I don't think we should be

using an individual daily peak which is driven by rare and unusual events and with long acting compounds don't have the ability to have that immediate punctate reaction or impact on the system.

On the other hand, using the mean itself is probably a little bit too -- not conservative or not conservative enough, because if we look at this for our mean over this last month is .8, and we're really driving right up to a level that we would regard as -- we would start regarding as dangerous.

Certainly, if the mean were 1, we would be over that danger level, and we probably wouldn't want to do that. So the mean isn't quite the right answer. The mean exposure, the mean daily exposure over that 30 days is not quite the right answer. It's got to be something less than that to be safe.

Exactly how much less it should be, I think, kind of relates to the inherent variation, how often excursions and large spikes and unusual events can occur; and we've got some ideas about how to approach that.

My point in bringing this out here is to stimulate folks in the agency to think about the right way to solve this problem. The analyses I've done suggest that the right answer is not to use the mean

but rather the mean plus a fraction of the standard deviation, and the fraction of the standard deviation -- the amount of the fraction depends upon the individual kinetics, the individual half-life of the individual compounds in the risk cup.

In fact, that sort of analysis works, and it would be a useful way to go about protecting the -- both protecting the public and preventing the analysis from being more conservative than it needs to be.

So to summarize here, we've talked about the process of dose normalization. I've urged you to recognize the kinetic difference among chemicals in a risk cup can be as important as potency differences.

Simply because we're normalizing compounds according to potency doesn't mean they are equivalent compounds. Very large kinetic differences can occur. That's especially true if we're dealing with something like carbamates which have a half-life on the order of 30 minutes or 45 minutes, and adding them in with something like OPs which have half-lives on the order of 100 or 150 hours.

So we need to be very aware of those kinetic differences and factor them into the analysis. We need to select the correct time frame for the compounds in the risk cup, and my suggestion is to use

something like four half-lives of the longest acting compound in the risk cup. For a group of compounds like OPs, that would be on the order of four weeks.

Finally, use a realistic exposure metric that accumulates temporally separated exposures in a manner consistent with the kinetic properties of those chemicals. Thank you very much.

(Applause.)

DR. NORRIS: Do you have some questions? The other thing we can do is briefly have all the speakers come up and start the panel discussion now, since we are running a little bit behind. Then if you would just stand right there, you can ask your question when they get here.

Okay, we are ready. Thank you.

QUESTION: The example you used where you were looking at the cumulative risk from the OPs, you used red blood cell cholinesterase inhibition. I think that people would generally agree that cholinesterase inhibition and toxicity is perhaps measured by other parameters, signs; and other anticholinesterase type -- or cholinergic symptoms is not necessarily correlated with the red blood cell cholinesterase inhibition.

My question to you is: You had compounds

there from your company, and I'm just wondering when you ranked -- You ranked the potencies -- With what you did, you ranked potencies by cholinesterase inhibition. But if you looked at the same chemicals in terms of what you know about their toxicity in terms of clinical signs and cholinergic type responses, were the rankings similar or did you find big differences in potency if you would look at toxicity by that other way?

The reason I'm asking that is because, of course, the agency is going to have to look at risk mitigation issues, and we are going to be getting chemicals from different companies. So one chemical could emerge from a process such as what you have looking very bad in terms of cholinesterase inhibition but not necessarily seeming to make any sense by other criteria of toxicity, i.e., clinical signs and that sort of thing.

DR. CHRISTOPH: Yes. I focused on red cell cholinesterase inhibition, because that's where the agency seems to be going. My personal opinion is that we should focus on brain cholinesterase inhibition, but that's just what I think. It just makes more sense to me, because basically we're talking about red cells, and essentially the red cell

cholinesterase inhibition is really a biomarker of exposure, to me, more than an adverse effect.

The rationale, of course, is that it's correlated with an adverse effect elsewhere and is a surrogate for peripheral cholinesterase inhibition. I think Stephanie would be a more appropriate person to comment on that.

I really think -- You know, there was, of course, a huge discussion about what's adverse in terms of cholinesterase inhibition, and I think the agency has just decided that it is cholinesterase inhibition itself that is. In my mind, the thing that's adverse about it is not inhibition of the enzyme. It's the consequences of inhibition of the enzyme, the consequences of the kinds of things you were talking about, signs.

Well, in fact, those are fairly insensitive. The things we use to measure those are our eyeballs, for the most part, and the kinds of things that would occur that are pretty easy to see up to really high concentrations in high levels of cholinesterase inhibition, things like tremor and so on, are simply not particularly sensitive instruments to measure those kind of things.

In my mind, the real adverse effect is

disruption of the fidelity of neurotransmission. That's kind of the bottom line on this. At what point is there so much acetylcholine in the synapse that the fidelity of neurotransmission is degraded?

That endpoint, no one is measuring, to my knowledge. I guess that's -- I would like to see work focused on that kind of problem.

So the nature of your question was how do these other kinds of behavioral things correlate? In fact, we really haven't studied that very much or studied that particular question very much, because, frankly, it's not important to us from the context of regulations. The agency drives the regulations, and the agency has decided that red cell cholinesterase inhibition is where we're going to be driven.

So I'm sorry that I can't offer a more detailed answer than that.

(INAUDIBLE QUESTION)

DR. CHRISTOPH: I really can't answer the question in detail. I'm sorry. I don't know.

QUESTION: Yes. I think, for the most part, those of us who are in the business of the risk mitigation part of it, and not to cloud the issue, but when you speak of that one in a million individual with higher levels of sensitivity or one in 1,000, if

we break it down even further and we correlate or we use an example of children one to six, I think for the most part for us, we try to regulate on the side of safety.

I think our greatest challenge is to make sure that we're using sound science. But by doing that, if you take that one in a million individuals and, you know, we use that age group of one to six, just for example, I think we are probably in a -- you know, it's safer to make sure that we are regulating on the side of safety for that particular age group for the various reason of, in general, the chronic effects over the long term that they could be exposed to various chemicals, OPs in particular.

So that is one of the things that, you know, we primarily try to keep in mind when we look at mitigation for various OPs.

DR. CHRISTOPH: If I can could just comment on that, companies are not interested in poisoning people. We have the same interests you do in terms of ensuring the safety of our products.

Throwing around figures like one in a million -- I actually believe we are talking about those kind of risk factors here with the use of pesticides. That's my personal belief, as I've kind

of gone through and looked at it. There are certainly people who would disagree and think that the risks are much higher than that.

You are absolutely right that the highest risk group that always falls out of all these analyses are children one to six.

QUESTION: I have got an anecdote. In a former life I was a Food and Drug inspector, and a bunch of us were sitting around sniggering one day about how we were going out and looking for swordfish based on numbers, mercury numbers, that had to do with eating swordfish every day. That was in Massachusetts. Swordfish was a luxury good.

Then one of the women in the group looked sort of stricken. She was on some kind of diet. She was eating tuna every morning -- every evening. That was her big thing, was tuna.

My guess is you're sort of assuming that the 12 apple person -- eating 12 apples is like being struck by lightning, and I think that's wrong. I think that people who eat 12 apples one day are going to eat 12 apples the next day. This is someone who has a serious apple thing, and you do have to think about that person in the course of that risk assessment.

DR. CHRISTOPH: I agree with that. I would just like you to think about the transition rules. You know, there may be an individual there that eats 12 apples day after day after day. How frequent is that individual? You know, think about that when you build a Monte Carlo is my point.

Sure, build that person into the analysis, and build that person into the analysis with a frequency that they actually occur in the population. I don't know exactly what the day to day transition rules are in the Monte Carlo. I mean, it's a complicated question, and there are some data from three consecutive days of people, real people, eating food. That can certainly help in building that one month of exposure.

Something like the three-consecutive day data in the database would help us get to understand how often that person eats -- how frequent that individual is that eats 12 apples a day.

DR. NORRIS: One more question. Thank you.

QUESTION : Okay. I had a question about your normalization of data. If you were using two compounds that were not equally efficacious, would you use the same methodology to normalize data or would

you have to adjust it, since the ED_{50} would be somewhat different?

DR. CHRISTOPH: Well, I think the whole point of normalization is that they are not equally efficacious. They differ in potency or differ in efficacy. Is that what you mean by efficacy here?

QUESTION: Well, in terms of efficacy, I mean their ability to induce a response. When you presented your example of how you would normalize data, both of the compounds appeared to produce the same amount of effect.

DR. CHRISTOPH: right.

QUESTION: See what I'm saying? So if you were using two compounds that did not produce the same strength of effect, how would you normalize the data then?

DR. CHRISTOPH: So your idea, one compound would be from zero to 100, another compound might be zero to 50 and not go any higher than that?

QUESTION: Correct.

DR. CHRISTOPH: In that case we are dealing with a case of non-parallel dose response functions, which bring us into a whole special category of events.

I think the first response when you end up

with non-parallel dose response functions, you should ask are these really compounds that belong together in the same risk cup? Are they truly common mechanism chemicals or is there something different about them that makes them work real differently?

There may be mathematical ways to handle that. One way to think about it is, since we are really concerned about the low dose side of this equation anyway, is the lower half of the dose response curves -- are they pretty similar to each other? And if those are pretty similar to each other -- We can forget the stuff that happens above 50 percent cholinesterase inhibition, because we are not going to drive any human to 50 percent cholinesterase inhibition by this process, by eating food.

So you know, if it's that case, let's deal with the lower half of the dose response function, see what's going on down there. Can we somehow convince ourselves that the lower halves of the functions are superimposable after a normalization step?

DR. NORRIS: Greg, I didn't mean for you to end up having to answer all the questions, if anybody else has anything to say. I would like to add that I think, if I ate 90 apples in one day, I would have other problems.

Well, let's go to lunch. If anybody is not from around here and needs to know where to go to lunch, apparently, I've been told there's restaurants all over. I can see the pesticide people saying, yes, there are restaurants everywhere. I hope we'll find them.

Thank you. We'll see you back here at two o'clock.

(Whereupon, the foregoing matter went off the record at 12:41 p.m.)

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AFTERNOON SESSION

Time: 2:05 p.m.

DR. NORRIS: All right. Most of us are back. Others will probably come trickling in. If I can have your attention, please, we'll begin the afternoon session of our program.

Beverly and Ethyl, I've been asked to change the name of our program by management again. They would like to make sure it's the neurotoxicity of chemical mixtures. Perhaps the management is concerned that there will be the neurotoxicities of the mixtures of the colors in my dress or something. I'm not sure. We aim to please.

We have four speakers this afternoon, and I'll introduce them all at this time and then briefly as they get up to speak.

Dr. Abou-Donia is Professor of Pharmacology and Cancer Biology and Professor of Neurobiology at the Duke University Medical Center.

Dr. Anthony Riley is Director of the Behavioral Neurosciences and Professor and soon to be chair of the Psychology Department at American University.

Dr. Rick Hertzberg initiated the EPA Mixtures Risk Assessment Research Program here at EPA

and chaired the initial Mixtures Risk Guidelines. He has also developed the first version of EPA's mixtures database, Mixtox; and I hope you're finding some more data to add to your database today, Rick.

Dr. Herman Koeter: Dr. Koeter is the Principal Administrator at the Paris based OECD Environmental Health and Safety Division. He is in charge of test guidelines, harmonization, the endocrine disrupters program, and the animal welfare policies.

We are very proud and pleased to have all of you with us this afternoon. Thank you. I won't take anymore of your time. I will let Dr. Abou-Donia begin.

DR. ABOU-DONIA: Thank you, Dr. Norris. My talk this afternoon is on chemical/chemical interactions which we have been hearing about all morning. As we know, most individuals are exposed to multiple chemicals. We are not exposed to just one chemical.

FDA requires testing of drug-drug interactions. The testing that the FDA requires actually is very intensive, and they require particularly the metabolic profile of each interaction, including the specific enzymes of

cytochrome P450 and the effect of each chemical on the induction or inhibition of the enzyme as well as of the MDR peak glycoprotein, the drug transporter in the body.

Unfortunately, there is no requirement for drug-pesticide interaction or pesticide-pesticide interactions neither by the EPA or the FDA. When we think about it, chemical companies discovered many years ago that combined exposures to chemicals is more toxic. That's why it's very rare that anybody would use one insecticide in the field. Usually, it's used much more than one insecticide.

On many occasions I have asked the questions to some of my friends in the chemical industry, do you have any data on the effect of combined exposure and health effects? They said no. Why? Because this is not required. So I think this is an area that should really be -- should be looked at.

The other thing is drug-pesticide interaction, which would be a joint venture between the FDA and the EPA. All of us are exposed to pesticides, one way or the other, and we are also exposed to drugs. We have prescription drugs and over-the-counter drugs, and we get exposed to both of

them at the same time, but we don't really know if this would cause any interaction.

That's why our specific -- our study was to investigate the interaction between a drug, which is pyridostigmine bromide and insect repellant called a DEET, and an insecticide, permethrin. These three chemicals were presumably present in the environment during the Gulf War, and they might be involved in the Gulf War diseases or illnesses.

The pyridostigmine is a carbamate that has positive charge. So presumably, this carbamate does not cross the blood-brain barrier. It only acts on the peripheral system. Pyridostigmine bromide is used - - is actually approved for use for myasthenia gravis at very, very large doses that range between 200 to almost 1,000 milligrams per kilogram dose. However, it is used -- It was used during the Gulf War as a prophylactic treatment against possible death by nerve gas.

The way it acts, as we know, it will shield the acetylcholinesterase in the peripheral nervous system so that when there is exposure to certain of the nerve gases, there will be protection; and then when the gas goes away, the enzyme will eventually spontaneously recover, and the person will

survive.

This chemical is fairly toxic. It has a LD_{50} of 61.6, which is less than half of that of the chlorpyrifos which you have been talking -- hearing about today. It's about 150. Of course, this is a reversible inhibitor of the cholinesterase.

The other chemical which we have been using is called DEET, which is -- This was developed in 1940s by the military to be used for military personnel when they go in the tropical areas. However, it's now available in the market, and an estimated 50 million Americans use it every year.

This chemical is used as an insect repellant and has very, very low acute toxicity, 3,000 milligrams per kilogram. However, in the literature there are several reports of death resulting from exposure to DEET. So even though it has very low toxicity, it is still lethal at certain dose levels.

The third chemical we have been using is permethrin, which is -- This is a pyrethroid insecticide, and it acts by disrupting the sodium channel and the axon. Now this chemical is even less toxic. The LD_{50} is 9,000 milligrams per kilogram in rats. However, the permethrin is very widely used for human use.

It is used in many shampoos for treatment of lice, for control of lice. It is also used to impregnate carpets, mattresses, many of the linen blankets. For those people who are actually sensitive to mites and other insects, that would reduce some kind of allergy.

So this chemical is very widely used. During the war it is estimated several hundred thousand American personnel used uniforms that were impregnated with permethrin to control insects. So potentially, a soldier that was in the Gulf War might have been exposed concurrently to pyridostigmine bromide, DEET and permethrin at the same time.

Some years ago, 1994 or '95, we did some study by actually trying the effect of each one of these chemicals alone at a very high dose and then in combination. With the high dose, that caused no effect. When we used them in binaries, unlike single, there was minimum toxicity. Binary combinations was greater toxicity. When we used three chemicals together, they produced paralysis and sometimes death.

Later we applied for a grant from the Department of Defense, and we are using the same combinations to study their action at a real life constant dose level that presumably the soldiers were

exposed to.

In this study we are using -- we are actually studying both stress as well as chemicals. the chemicals that we used are the pyridostigmine bromide, DEET and permethrin.

The dose that we are using -- These are the doses that we obtained from DoD. The dose was 1.3 milligrams per kilogram. This is the exact dose that the soldiers were taking. They were given -- They were supposed to take three 50-milligram pills a day. That's 90 milligrams, which divided by 70 would be 1.3 milligram per kilogram per day orally.

DEET, they told us the dose was 40 milligram per kilogram dermally. So we used that amount. Permethrin, .13 milligram per kilogram in ethanol dermally. We treated the animals, rats, Sprague-Dawley rats, for 28 days.

Also another group of animals was given stress by simply placing the rat in a plexiglass restraint for five minutes every morning. The design of the experiments was like this. We have four groups. One was chemicals, the three chemicals, the same doses I just gave, and stress, chemicals under stress, and the control. Controls were given water orally as well as ethanol dermally. The water was

given at the one milliliter per kilogram.

So what we did, we had male Sprague-Dawley rats treated 28 consecutive days. Animals were weighed weekly. We had subgroups of five rats that were used for enzymatic analysis, to analyze for brain acetylcholinesterase and plasma acetylcholinesterase.

We determined the binding of m2 muscarinic acetylcholinesterase muscarinic receptor to [3H]AF-DX384. Now we determined that the integrity of the blood-brain barrier, which we heard some about it this morning from Dr. O'Donoghue -- we determined that a similar way -- a couple of ways.

One, we determined the uptake of triturated hexamethonium iodide, a chemical that has positive charge, is not supposed to cross the blood-brain barrier. The horseradish peroxidase as well was used in this experiment. Then we looked at the histopathology in the brain as well as the liver.

Well, this treatment for 28 days resulted in -- There was not clinically different -- The treated animals are not much clinically different than control. As a matter of fact, they might have done a little better, because they were a little bit lighter or gained less weight.

The weight gain was -- All of the animals

The diesel here was not -- let's see. It was not used in the calculation of the line. It's an actual data point, but the line is based upon the other mixtures of PAHs.

So in this case, it looks like there is some consistency, some proportionality between mouse skin tumors and human lung cancer risk. That's kind of the basic idea here. If you have a way to scale up from in vitro assays, demonstrate it, and then as we did with the interaction patterns before, you have a way to sort of generalize for untested chemicals or untested mixtures.

Another revelation we had, as I mentioned briefly, in looking at the data and the published studies was too many definitions of interaction, too many definitions of additivity. So being a regulatory agency, we have the power to make our own definitions. So we decided, first of all, to simplify.

That was to have dose addition as our default, as our no-interaction, so we don't talk about additive interactions. We just talk about dose addition as no interaction. Anything higher than that is synergism. Anything lower than that is antagonism.

That made it extremely simple, and in a

regulatory sense it's very useful that way. We don't need to know the other things. We just need to know, if we're making a mistake, which direction are we erring in -- on, toward?

I think I'll move on. Another thing we found in looking at the data is that synergism wasn't reported that often. We thought that, since academics do most of this kind of research, you need to publish. You need to publish positive results.

So you would want to have a selection of chemicals that would show you some interaction. Surprisingly, it did not happen.

When we looked at trying to estimate magnitude for the handful of studies that were actually useful for doing this, we had high dose interactions of tenfold to twentyfold or more. We had low dose interactions that were two, three, five, in that range.

So our default so far for our mixtures guidance is a magnitude of five. It's a fivefold decrease in effective dose, if you're talking about synergism.

This is the hazard index. It's the basis of dose addition. Superfund has this in their guidance. The bottom expression is what's used for

the dioxins where you replace the mixture by its surrogate or index chemical, the equivalent dose in that chemical. It's the same formula, but you translate to each other.

We had one improvement, and this is where I'll give you some more lessons learned. In '92 we came up with an approach to replace the conservatism in using the reference dose, the reference concentration as a scaling factor for relative potency.

The problem with those is that they are bounding estimates. They are supposed to be the -- representing the critical effect. If you are trying to do some common mode of action that is some other effect, then you are going to be over-regulating or overestimating the risk, because you're using acceptable doses or dose scaling that's way, way too low.

So we could just do the same kind of process but do it for the effective concern. If we're looking at neurotoxicity, then make sure that all of these pseudo reference doses are now based only upon neurotoxicity data. Then you don't have the over-conservatism.

Well, so we wrote this up, and we got

slammed hard by our regions and our program offices, because EPA has no procedure in place for developing these target organ toxicity doses. So why advertise this as a great new method if we can't do it? So that's my lesson learned number three or four, I guess, by this point.

Good ideas need to be published, but if you put them in guidance, then someone expects you to actually do it.

Now once again we're back in the case of not having enough information. Here you see again a lot of places, not as many as we were concerned about, but you see that in many cases we don't have enough to do a TTD so we can resort back to using the reference dose. Okay, we have five or six cases here where we are essentially going back to defaults.

So a good approach. Just doesn't have enough information to do it.

This was raised several times. So what do you do if you do have interactions? How do you account for them? We talked to a lot of people who do interaction studies. We looked at a lot of literature on pharmacokinetic modeling and mechanisms of interaction.

It seemed that we had some general

principles that seemed to come out. One was something that, if you didn't have your chemicals at equally toxic doses where one starts to dominate, then the interaction often starts to die off. So we would want to have some kind of way to reflect that, and that's what this function does down here. As the chemicals get more dissimilar, you have a curve that goes like this. One chemical becomes more dominant. The interaction goes down.

These are just general qualitative characteristics. What we have here, basically, is this part over here is the hazard index, dose addition, and we just took every hazard quotient and modified it by a function that represents the interactions to that chemical.

So this is all based upon pair-wise data. That's weight of evidence considerations, or the Bs right there. So this is a way we can kind of incorporate the data we do have on interactions, quantify it, end up with a change in the risk estimate.

What we need is more information so that all of these functions in here can be replaced by real dose interaction/magnitude relationships and not just sort of plausible relations.

Okay. This just kind of explains the different pieces.

So here's my summary of our lessons. First of all, we have to be flexible in our methods. We can't require testing or new data for every mixture we encounter. There are too many mixtures and too many varieties of exposure situations, changes in the proportions, in total dose.

You saw the influence of changes in sequence, changes in repeated exposures as opposed to a single shot. All those things have to be somehow reflected. When you have a mixture and you're trying to do something to build up from component information, the component information is not the same for each chemical.

So that's what I mean by flexibility. You have to have a way to use the different varieties of information.

We don't have a lot of experience yet with mixtures. Superfund is our only main office that's done this for any length of time. So approaches such as the acceptable daily intake that ran FDA and EPA for a long time and build up a history, we don't have that with mixtures. So we don't have a lot of procedures set in concrete yet that we can rely on as

passing the public sector.

This is some examples of the problems of multiple data types and qualities. This is one of the concerns, I know, with OPP when they put out their guidance, was what do you do when you have one chemical that's just dominating the whole mixture assessment because it has a huge uncertainty factor.

I haven't heard whether that got resolved, but there's some nice suggestions on how to fix that. So I'm looking forward to the next version to see if it's included in there.

Incorporating interactions: There are lots of pairs. A mixture of ten chemicals has -- what is it, 1,000 pairs or so? We can't information on all those pairs. We know that. So you're going to have to have these data gaps and have some way to fill in those holes with something.

Then this is typical of the government, is that you will have defaults so you can make a decision in spite of lack of data. When you have defaults, you have to combine it with real information. How do you do that in a nice, scientific fashion without having the defaults just dominate everything?

If they dominate, then there's no point in gathering the better information, because it no longer

plays a role. We found this with NOELs, doses based upon NOELs.

If you have other chemicals that have really good ED_{10} or ED_{01}, it doesn't matter a whole lot, because the uncertainty surrounding the NOELs is so much higher that it just swamps the extra accuracy that you get from the other chemicals that have ED_{10}.

We are all hoping we can move toward pharmacokinetic modeling, but we need to have human data. There is already a lot of questions being raised about overuse of PBPK models based only on the rat. So we need to have some better validation, I think, there. As I said before, interaction magnitude -- we're really in the dark there.

Okay. We do have a lot of good ideas. It's really exciting for me to work with the Pesticide Office and watch them go through their haranguing to put together guidance after we spent so many years and still hadn't had any new guidance come out.

A lot of new ideas came out. There are a lot of ideas just today. A nice advantage, I guess, of being one of the last speakers is you see many new concepts being put forth, but to have them in their practical method, a guidance for an agency to use, we need more, and we have to have ways to know what to do

when those pieces of information are not present.

The bottom line -- It's still there. This is the one we had hoped for 15 years ago. So any of you that have any insight on how to do these studies at the really low environmental levels, start showing us where these thresholds occur, where below that level we don't have to worry about interactions. It would really make our job much, much easier.

Thank you.

(Applause.)

DR. NORRIS: Are there questions now? Thank you.

Our next speaker is Dr. Herman Koeter, and I'll make another announcement after that. Thank you. What I'd like to suggest is that, in lieu of taking a break and then coming back, if our speakers wouldn't mind, if we could talk to you for a few minutes during the break over cookies and soda in the back room there. We can skip the panel discussion and just have a more intimate conversation in the back over our break, and then we will excuse ourselves from there. Thank you very much. Now we'll be set up here in a moment for Dr. Koeter.

DR. KOETER: Thank you very much for the invitation to speak to you here today, and I consider

that a privilege to have an opportunity to explain a little bit more about the work of OECD and, in particular, with respect to the work that we do on the classification of mixtures and the development of test guidelines.

I apologize for the sort of prehistorical way of presenting my information. The slides that I use are -- It only needs a bit of focusing. Here we go.

Well, first of all, the OECD, as some of you do know and many of us probably don't, is an organization for economical cooperation and development, and the E for economical is quite an important one. What that means actually that we try to improve the economies of our member countries, and that's the main goal of that organization.

Now you may wonder what chemicals have to do with economy in that respect. By the way, these are the current members of OECD, and I say current because, well, there is sort of a continuous debate on the adherence. A lot of countries do OECD. The ones that you see up here, some of them have been members since the early sixties when OECD was established. Others like Mexico, Korea are members that joined the organization only recently. That means in the last

couple or four or five years.

As I said, it's an economic organization, and chemicals do play an important role in such a way that we feel that the management of chemicals in member countries, if we could harmonize that in one way or another, would save a lot of money, and we published a document on that that shows that slowly, because of the work done by one division on chemical safety and the harmonization of methodologies, that that would save a numerous amount of dollars. As a matter of fact, it's around approximately $56 million in OECD member countries on a yearly basis just because we share information between countries. We share registration procedures, and we have harmonized testing methodology.

As you all know, the risk assessment paradigm consists of the hazard identification, and from there on further down to risk assessment. In our work on harmonization of classification, I would like to start to show you what role mixtures play in our work and to give you a little bit of an impression how much work it is to harmonize certain things.

We identify these steps here, the hazard identification steps, which we would like to harmonize between countries and among countries, and then after

that and based on that hazard identification, the classification of chemicals and mixtures, for that matter and, when that is done, the hazard communication.

Well, the latter part is a part where we share the work with other international organizations such as WHO and ILO. You can imagine that communicating a certain hazard and the detail of a hazard to people that are illiterate, live in countries where cultures are completely different from western countries or from OECD member countries in that respect is quite a challenge in itself.

Well, in order to organize that work, we started to establish a comity, a task force, we call that. Unfortunately, in OECD we have rules and regulations what you will call a group, whether that will be a working group or a task force or whatever. But that is very clearly defined, and a task force is a relatively higher level. That means there's most definitely a policy component to that as well, not only technical.

Here you see the task force that we set up for the classification and labeling work. It consists not only of member country representatives. They are all nominated by their governments, but also the

European Commission representative. That is in addition to members from the European member states.

We have chairs from other groups that do related work. You see the task force of NCs. That stands for national coordinators of the TGP, which is the test guidelines program, and I will talk a little bit about that in a minute.

We have the chair of our working group on pesticides. That is a working group that works on the harmonization of pesticides work. Then we have a whole lot of acronyms that I will not explain, but they all deal with international organizations that, in one way or another, are involved in classification. You can imagine, classification is not only for consumer use and for pesticides. It's also used in transportation quite a lot and in marine transportation, the big tankers, it makes a really big difference whether their contents is classified as hazardous or nonhazardous, and within that hazardous category also further details.

So IMO, International Marine Organization, transport of dangerous goods, U.N. organizations. BIAC and TUAC stands for the official organizations that represent industry. That is BIAC, Business and Industry Advisory Committee to the OECD, and TUAC is

worker unions, international worker unions. They all play a role in that work, and they all disagree usually.

We recognize a couple of steps to sort of facilitate and organize the work, because we deal with classification systems that are around, that are substantially different between countries, and we first thought, well, as a first step we need to know what exactly do these classification systems look like in the various member countries. Where do they differ, and why do they differ, and do they all work in the same way if you would compare them, run chemicals through one system into another, do you end up with different classification and so on and so forth. That is step one, and we call that a detailed review document.

These documents can be substantial, usually 100 pages or more where it has a clear comparison between all the systems.

As a next step, we would then propose a harmonized system which is based on existing systems. We had no intention to develop new systems. Although some of the experts would really like to take that opportunity and say, well, we've learned from the past, let's develop things again, we try not to do so.

The reason for that is that we want to avoid that after you have developed a harmonized system that everybody has to classify everything that had been classified before. So we would like to minimize the changes in classification that would occur once you have adopted the harmonized system, which is very difficult in itself.

Step three is then the discussion that would fall out at the various levels, the technical level, policy levels, and so on.

Step four would be the endorsement, and that is an official endorsements by the governments of the OECD member countries. Since we have only 29 member countries, together producing still 85 percent of all the chemicals in the world, we want to reach out further.

That's why we have sought acceptance in the U.N. system and the decision making system there in the U.N. which we will do together with other international organizations and especially those that are also involved in the hazard communication. That means in that center part which you see there, IOM stands for the International Organization of -- well, I forgot it myself for the moment. It's an international group of organizations that, like U.N.

organizations and OECD and the Commission would all work together and to bring that together to U.N., to ECOSOC where it will be adopted in the near future.

Well, where are we today? That is the sort of framework in which we work. We have identified these endpoints that you see up there, because classification systems, most of them but not all, include all these endpoints in one way or another.

You see, the ones that are listed at the upper part are all endpoints that are considered, and most of them are real, true endpoints. The last one, chemical mixtures, is a different entity. We have added that, because currently do there exist specific classification systems for mixtures that are different than for chemical substances.

There are also a couple of endpoints that are not covered in any existing system, which are neurotoxicity, unfortunately immunotoxicity, and then a couple of smaller hazards. We call them smaller hazards, because they are not really interesting for all the member countries, like water activated toxicity such as in contact with water would vaporize and, by that, cause a hazard, aspiration hazards, hazards of defatting agents, and so on and so forth.

In order to deal with all these different subjects, we need different groups of experts. We cannot just put all the experts together and say, well, let's talk about that, because we need different experts for these different groups.

So we have established groups of experts on acute toxicity, mutagenicity, repro-tox, all these different things. These groups came together to discuss their various endpoints and to reach consensus or to fight about that.

In that last slide you saw one group on chemical mixtures, and I will not go into the details of the work that we did on substances, because it's not so much of interest for this audience today. So I will focus a bit more on how we dealt with mixtures.

One of the things that surprised me today, being the last speaker -- we've heard many speakers, and nobody really started to discuss what is a mixture. Well, Debbie did it, to some extent.

Well, there are a lot of people that would say, yes, that is a mixture. A mixture is now being considered, at least as a working definition in OECD, as a mixture of two or more chemicals that do not react. Especially the latter addition is an important one, because you can put chemicals together and they

react. Well, that can become a mixture after the reaction process is done, then fine. Then you have a mixture that will no longer react, and that is what you work with.

Indeed we have spent meetings talking about where does it stop. Is this table a mixture? Is the dress a mixture? These are all part of that definition. We had to stop that discussion and say, well, we all sort of understand what a chemical mixture is, and let's don't really bother about that. So we left it aside, but we consider it all in the sort of the general way that we in our various legislatures and regulations consider a mixture.

The expert group, all in the classification for mixtures, we have extended -- expanded our group beyond OECD where you see countries here like Brazil, because we felt that classification of mixtures is especially important in developing countries where a lot of the chemical -- not so much the production of the chemicals, but the preparation of mixtures is being done. Products are being made, and people are exposed to large extent. We want to have specific input from those countries as well.

You see, the group is more limited here. We wanted to have a sort of regional representation

from the southeast like Australia, Canada and Mexico, then also to sort of represent developing countries; two people from EU member states. There's a couple of countries that alternate on that, and then we have the U.S. I think I forgot to mention the U.S. on that. The U.S. is on it definitely, the European Commission, Brazil, BIAC, TUAC, and a couple of other people.

The branching of the tree continues, because below that group there are again subgroups that deal with the various endpoints that are of importance for mixtures.

Of course, in principle, all the endpoints that I listed before for substances are also being considered for mixtures, but it was not considered necessary to have separate groups of experts for each of the endpoints, because we have dealt now with substances, and I didn't mention that before. But that is the past station. We have reached agreement on that.

So using the harmonized classification systems for a particular endpoint like sensitization and dermal and eye irritation and corrosion, people felt sufficiently confident as a bigger group to work with that and try to harmonize that.

The ones that are listed here are the ones

that are considered difficult. Acute toxicity has been and still is extremely difficult, how to deal with that.

C&M stands for cancer and mutagenicity, and reproductive toxicity are also very difficult. Environmental hazards are still in its infancy anyway, the classification for chemical hazards to the environment. Then we have a separate group that tries to put this altogether into a proposal.

Again, I will not focus too much on all these endpoints, because I can spend hours just talking about that.

One thing that struck me is that within the discussions or part of the discussions, one of the biggest differences in all the discussions about mixtures is that additivity of effects is considered a very important aspect in Europe.

In U.S. it's not considered an important aspect. I just learned this afternoon, at least in EPA, in your guidelines that are coming out for mixtures, additivity is considered. But as you understand, in this international forum we deal, of course, with EPA, but we also deal with FDA.

We deal with your Department of Transportation. We deal with OSHA. We deal with

CPSC, and all the other agencies, they don't want to be bothered about additivity. They have a classification system for mixtures which is based on the individual hazards, and they just apply sort of cutoffs for each of those separate hazards and don't add them up.

In Europe they have very complicated systems for additivity, and they do not -- Well, they do consider also synergism, but they do that in a sort of a, let's say, expert judgment type of approach, which is more or less on a case by case rather than using formalized or other stuff, which they do for additivity.

This is just to show you the number of meetings that we have only on that particular group of mixtures in order to reach consensus. The seventh meeting is in parentheses, because we hope that next month at the sixth meeting we will reach final consensus.

You see also -- This is just to give you an impression how much work is involved here, that this is only the list of meetings of the expert group. All the subgroups below that are not listed, and then in addition we have more than 50 chemicals.

We've heard them already today, and

teleconferences is one of the nightmares that I sort of have, because I have teleconferences almost every day, if not every day, on all kinds of different subjects.

So where are we with mixtures today? Well, first of all, we have the data review document. This is available on the Web, and one of the slides that will follow this gives you the address. If you forget about it, it's just www.oecd.org.ehs for Environmental Hazards Safety, but it comes up in one of the slides.

Here we compare the classification systems that are existing today, and in addition to the North American from Canada and U.S. and the European EU, Sweden being a member of the European Union still has a classification system that's slightly different from the Commission.

As you may know, Sweden is one of the more recent members of the European Union, and still is in a period of transition, and now tries to get some of their specific aspects into this harmonized system, which they could not manage at the time they were becoming a member in Europe. But we also see that countries like Slovenia, Korea -- there are countries in the world where you would not have expected that

they have detailed systems for a chemical mixtures classification, and they have.

They have all been considered. We have compared consumer products, transport, pesticides, new chemicals, hazardous wastes, and the title of the document you see there below.

The step two proposal that we have now, the first one, and the second one is coming up in May, has these chapters: General consideration, building block approach -- and I wish I had more time to talk about the building blocks to what really constitutes harmonization, because harmonization is not standardization -- definitions, and then these endpoints are all part of that.

Here at the bottom you see target organ toxicity, and that is the whole on neurotoxicity today. It was considered by member countries officially that there was no need to classify substances specifically for neurotoxicity, no need to classify mixtures specifically for neurotoxicity.

What was agreed is that we would consider target organ as the home for that, and if we know that the target organ is the brain or central nervous system, we could mention that, but not have a special system for it.

Within mixtures we had this sort of ranking. We classify -- We have a system to classify whether the data are available for the mixture as a whole. You would say, well, that's the ideal situation, which is not always true.

There are countries, especially in Europe, that feel that with respect to cancer and mutagenicity and even reproductive toxicity, data on the mixture as a whole is not good data, because you have dilutions of the substance that can cause that carcinogenicity which will not show up in your testing when you test the mixture.

So we have a system where you would deal with when you have the data of the mixture as a whole. Then we apply bridging principles where we have additivity, synergism and that sort of thing included in rules and regulations.

Then we classify based on where we have no data on the mixture as a whole, but we have data on all the components. You can imagine that we say -- easily say all the components that, we have quite an extensive discussion, what is a component? How far do you go? Do you go to the .0001 percent contaminant in your mixture or do you have a cutoff? We did set a cutoff of one percent now.

Then we have classification based on when data are available for some of your ingredients, but not all. But this is just to show the complexity.

Finally, because I understand you're interested in neurotoxicity, and that's what I said where we have neurotoxicity data, we have separately identified two systems, one for effects of the single exposure and one for effects after repeated exposure.

These have gone back and forward many times, because they said, well, they should be all included into one system, both single and repeated exposure. Then we didn't manage to reach a harmonized system. So it was decided to separate them. Then we had them back again.

So we went back and forth quite a while, and we ended up having two different systems which have two classes today, and the first class is based on human data, basically, and the second on animal data. That's one distinction.

Also, the separation is based on the severity of the facts. The severity of effects, of course, can be related to the testing where the test methods for dose specific endpoints are considered important.

The criteria -- We have expert judgment

which is the leading thing. You can easily, well, that makes it pretty vague. Expert judgment in one country is very different from expert judgment in another country. But in order to harmonize even expert judgment, which is hard, we provide guidance of effects that are considered to support classification, effects that are not considered to support classification, and we have added also guidance values for cutoff of effects, which is currently used in Europe mostly.

Just one that shows a little bit where we have references to, neurotoxicity or at least it is made clear that neurotoxicity is definitely included there, like under criteria and like under effects supporting classification, we have statements there, significant functional change in central peripheral nervous system, including central nervous system depression and special senses.

These sort of syntheses are added scattered around in the document, and not only for the endpoint of neurotoxicity but also for many of the other endpoints.

A few minutes on the test guidelines development in OECD. That is sort of the foundation of the work on classification, because you need test

data. So you need test guidelines to do that.

These are based on what we call the MAD decision or the Mad Decision, which tells you that if data are generated according to OECD guidelines and in compliance with OECD GOP, practices shall be accepted in all member countries, which is today the case.

That means that, despite the fact that certain member countries, including the U.S. and Japan, for that matter, still have their domestic guidelines and even have different guidelines between agencies or within an agency, when a test is conducted according to an OECD guideline and then that OECD guideline covers the data required -- which, of course, that's a prerequisite -- this will be accepted by all member countries.

Just to give you a clue about how big that program is, we have currently -- Well, in 1981 we published our first set of 51 guidelines. Since then, the 11 addenda have been published with 90 new and updated guidelines altogether, and we have now a second edition of the work which was published in 1993, and it's now today available as hard copy or CD-Rom, and also online in our OECD bookshop.

This is the site where, if you go there, you'll find all the information on testing and

assessments and also on the classification. The publication are, unfortunately, a priced publication, but recently I've managed with the OECD management that they can also be bought now separately as guidelines, individually as guidelines rather than buying the whole set.

We distinguish various sort of groups that involve physical chemical properties, biotic systems facts, degradation, health effects, and special activities. You see a number of projects that are listed that we have today in our portfolio. We have one project on acute toxicity, for instance, which is a very large one. We have smaller ones. So altogether you have a bit of an idea how many projects we have.

Yo see that some projects are led by member countries. They take the leads. They organize the work. They have their experts providing first proposals for draft guidelines, the guidance documents. They could be member countries, could even be the industry.

Who do we involve in the work here? We involve not only the member countries, but we have partner organizations, the European Chemicals Bureau, ICH, which has been responsible for the harmonization

of pharmaceutical guidelines; IOMC, mentioned earlier, the combination of international organizations; IZO standards; industry organizations; and then we have input from academia, government and industry. All these things come together finally in a proposal at the Secretariat where we try to deal with that.

I will now use only two more minutes, if you don't mind. I will skip the procedural part, how we develop. I will just show you one example of neurotoxicity, just to see how much time it sometimes takes to indeed develop a guideline.

It started in '87, our Guideline 424. For those who know, that guideline started in '87 as a combined proposal from the U.S. and the Netherlands for a neurotoxicity test. After formatting, language changes, and so on and so forth, in '88 we can circulate that for review.

Well, you see, we had a follow-up meeting here in Washington, an expert meeting in OECD where we discussed the guideline, including the comments that were received. After review by international experts, and just besides, national experts means we have a database of about 6,000 experts that are all copied on guidelines, and that means that we do receive a whole lot of responses and comments, and in the Secretariat

we try to deal with that, put it together and revise guidelines accordingly.

In '92 we had another consultation meeting where we -- neurotoxicity, the relationship between that and the guidelines that we have for systemic toxicity was discussed, and in 1994 we were finally able to circulate a new proposal.

Another meeting was needed in Ottawa to further discuss comments, and some of you in this audience have been present at these meetings. In '95 we revised it again. In '96 we were finally ready to bring it to the policy level, and that means we were not really done there, because after the experts finally agreed that this is technically a good guidance or a good guideline, then policy people are considering it.

They look at economical, social impacts and welfare sort of things pressuring countries, and finally it was adopted in 1997. So that took exactly ten years.

It's one of those examples -- I think I stop with my overhead. It's one of those examples that I use when I say, well, if it takes more than ten years, then this is sort of a continuous process, because after about seven, eight years of discussion

among experts, you see a new generation of experts coming in, and that is very serious; because a new generation has different views, disagreements -- they are elderly people, and they come up with new ideas.

You start over all again in the discussion. So we feel that we have experience at this. If it takes more than ten years, you better stop it and start all over again, rather than continue.

So we were happy that we could finalize the neurotox in ten years. I hope that developmental neurotox which we started just a few years ago in '96, so to speak, and I have another series of slides on that but because of time constraints, I will not show them to you -- that we will be able to manage to finalize that guideline within a year's time from now.

I think I'll stop here, Debbie. Thank you very much.

(Applause.)

QUESTION: This may put you on the spot a little bit. In October the Netherlands is having a meeting to work on mixtures guidance or some mixtures approaches, I guess, for their country.

I would love to ask them a question about how that fits into what you are doing. How can I ask

that question?

DR. KOETER: Well, you can ask that question probably to address it to me, although I could not give you that answer right away. Unfortunately, member countries do not always share information about their national events with the Secretariat.

I think -- I'm not sure whether that meeting that you refer to really deals with classification of chemical mixtures in the same way that I was talking about that. If that is the case, then, well, at least the Netherlands should inform the Secretariat. So if you wish, I can ask them and send that information to you. I do not have it here at hand.

MR. BLUOIN: Thank you very much, Dr. Koeter.

In closing, I represent the Technical Training Committee from OPPT. I want to thank all of you for coming to this, those of you that have made it through the day. The crowd has been diminished.

Special thanks to our speakers who presented us with a worldwide view of this fairly complex thing.

Special thanks goes to Debbie Norris, who

had this bright idea about a year and a half ago.

(Applause.)

Thank you very much, Deb.

Last but not least, thanks to all the people that helped put it all together, everybody from people that did the nametags, the people that are sitting out there, to Bev Sjoblad for hand holding us with the money and stuff like that. So thank you all.

There's cookies and stuff out there. I guess we have decided to not have the panel. So if we have some discussion, we can have it out there over cookies.

(Whereupon, the foregoing matter went off the record at 4:07 p.m.)

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CERTIFICATE

This is to certify that the foregoing transcript in the matter of: Symposium

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represents the full and complete proceedings of the aforementioned matter, as reported and reduced to typewriting.