U.S. ENVIRONMENTAL PROTECTION AGENCY

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SYMPOSIUM ON NEW SCIENTIFIC RESEARCH RELATED TO THE HEALTH EFFECTS OF TRICHLOROETHYLENE

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Renaissance Mayflower Hotel

Grand Ballroom

1133 Connecticut Avenue, N.W.

Washington, D.C.

Friday, February 27, 2004

8:11 a.m.

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VOLUME II

I N D E X

P R O C E E D I N G S

[8:11 a.m.]

DR. PREUSS: We are ready to begin the second day of the presentations, and at the end of the day today, we will have a panel discussion with all of the speakers hopefully participating, but they'll all be up there. I'm not sure they'll all participate.

And again, the same ground rules as we had yesterday: you will forgive me for repeating them. But that after each speaker, we will have time, I hope, for five or so minutes of clarifying questions, and at the end of every three papers, we will have a longer Q and A session, if there are still issues that need to be raised and talked about or so on.

At the end of the day today for the panel session, and I'm now speaking particularly to all the folks who have presented, I will ask you one question, and I don't want it to be a surprise, so I thought I would mention it now and give you a chance to think about it a little bit, that is,

given all of the things that you have presented, the papers that you have heard presented here today, which things would you particularly commend to our attention? That you all clearly know the field very well; you're familiar with this literature; you have heard the presentations, and again, for our purposes in moving forward with the risk assessment for TCE, we would appreciate any comments you have with regard to those kinds of issues.

At the end of that, I will also talk for about two or three minutes about where we're heading with this assessment, what our plans our and what our schedule is, so that all of you who are working in this field can have some sense of what is going to happen over the next months and year or two.

So with that, I'd like to begin the first session, and I'd like to call on Dr. JoEllyn McMillan from the Medical School of South Carolina, who will talk about TCE and hepatotoxicity.

DR. MCMILLAN: I'd like to thank the

organizers for inviting me to talk about my research. We are interested in the mechanisms of trichloroethylene hepatocarcinogenicity, but specifically, we are interested in the use of hepatocyte cultures to study aspects of this response.

Overall, our research goals are to determine the role of both peroxisome proliferation and mitogenesis in the development of neoplasia and what is the role of the peroxisome proliferator-activated receptor in this response as well, but more specifically what is the relevance of the events that occur in the B6C3F1 mouse to events that might occur in human liver?

As background for this talk, you have heard many of these before, the response is seen only in the B6C3F1 mouse and is thought to be due to two metabolites, trichloroacetate and dichloroacetate. For us specifically, we are interested in these two early responses, the mitogenic response and the peroxisome proliferative response. And the question is whether these

responses occur in humans and what relevance do they have?

This is a time line of the events that occur in the TCE-treated mice. Again, the early events, DNA synthesis and peroxisome proliferation; development of the enzyme altered foci and the adenomas and carcinomas. And what we're interested in is can these two early events be used as markers, or are they markers for the development of the later carcinogenic events?

So the question we asked, then, was can we use primary or hepatocyte cultures to study these two early events? And more importantly, can we use human hepatocyte cultures to replicate these events, and if they do respond in the same way, can the intensity of the response be used to assess the risk of humans?

First, do we see mitogenesis when mouse and rat hepatocyte cultures are treated with TCA or DCA? This is to orient you. This is a review of the in vivo response. These are our own data, but other people have shown it as well, that when

you treat this mouse with TCA or DCA, there is an increase in cell mitogenesis, and in our case, we used bromodeoxyuridine incorporation as an index of cell proliferation, and we saw an increase in incorporation at both 7 and 14 days of treatment with both TCA and DCA.

To study these events in hepatocyte cultures, we isolated rat and mouse hepatocytes by standard procedures; placed them in culture; treated the cells for 36 hours with the test compounds; gave a four-hour pulse of tritiated thymidine and then collected DNA and assayed it for the amount of radioactivity.

First, the rat hepatocyte cultures. Again, there was a low basal level of thymidine incorporation that we observed in these cultures, but when the cells were treated with a known growth factor, epidermal growth factor, we saw nice enhancement of thymidine incorporation, indicating that the cells are capable of responding to a mitogenic stimulus. However, when we treated the cells with varying concentrations of DCA or TCA, we

did not observe any increase in thymidine incorporation. We did not observe cell mitogenesis.

What about the mouse hepatocytes? The mouse hepatocytes were problematic in that they had a very high background level of thymidine incorporation. In fact, we could not induce any further increase in thymidine incorporation by treatment with EGF at any concentration. Thus, we could not assess whether TCA or DCA were mitogenic in these cells.

Back to the rat hepatocytes. It is well-known that hepatocytes in culture lose cells over time or hepatocyte cultures lose cells over time, and what effect would this have on the overall mitogenic response? So in the cells treated with EGF, we did initial cell counts, and then, after the 40 hours of treatment, we counted the cells again, and EGF prevented the loss of cells and actually increased the number of cells over those seen in the control cultures.

We observed an interesting phenomenon with

DCA in that it prevented this cell loss in a dose-dependent manner, although it did not actually increase the number of cells, so it seems to be maintaining the number of cells in the culture. This effect was not observed with TCA.

Another interesting effect we observed with DCA was an apparent interaction with EGF on the thymidine incorporation. Again, here is EGF increase in thymidine incorporation in a dose dependent fashion. DCA had no effect. When DCA was administered with both the low and the higher dose of EGF, there was a synergistic increase in thymidine incorporation. So the light purple bar is compared to the light green EGF bars, and I failed to put the significance on here, but these two higher doses of DCA were significantly different than the corresponding EGF thymidine incorporation rates, and the dark purple corresponds to the dark green EGF bar.

So there was--whether this interaction is due to a priming effect that is known to occur for some hormones and growth factors in that the

compound itself is not mitogenic, but when dealing with a growth factor, it can enhance the respon se to the growth factor or whether this is due to some other effect is currently under investigation.

This interaction was also observed when we did cell counts. Again, increase in cell numbers with EGF treatment; maintenance of the cell numbers with DCA and a synergistic increase in the cell numbers with DCA and EGF, and then these bs indicate that they are different from the levels in the EGF-treated cells.

So DCA appears to be acting in some way different from TCA in maintaining the cell culture, the cells in culture, and interacting with the EGF response.

What about human hepatocytes? We iso lated human hepatocytes from tissue that we obtained from organ transplant rejected livers, and we used this procedure of D'Ambrosio, Steve D'Ambrosio at Ohio State, to isolate and culture the cells. And this procedure allows the cells to maintain many of their liver-specific functions. And the cells then

can be used as primary cultures, or they can be subpassaged several times and still maintain their liver function.

So first, the thymidine incorporation. Again, when we have--this is cells from human, our sixth human liver sample. The primary cells again responded; there was a mitogenic response observed with EGF. So they are responsive to a mitogenic agent. As with the rat hepatocytes, there was no mitogenic response with DCA or TCA.

And the same effect was observed in the subcultured cells. This is the fifth passage of these cells. So the human cells act analogously to the rat cells in this effect.

What about the interactive effect that we observed in the rat cells of DCA and EGF? Again, a nice stimulation with EGF; no effect with DCA. However, the interaction that we observed in the rat hepatocytes with DCA and EGF did not occur in the human hepatocytes, either in the primary cultures or in the subcultured cells. So they do not react analogously to rat hepatocytes.

So, where do we stand? Can hepatocyte cultures be used to study the mitogenic response? Again, in rat hepatocytes and in mouse hepatocytes, there was no mitogenic activity of DCA or TCA. However, there was an interesting effect in the rat hepatocytes. In human hepatocytes, again, there was no mitogenic effect with DCA or TCA, and there was no interaction of DCA and EGF.

What about the next, the other early event, the peroxisome proliferative event? Can we replicate this in the hepatocyte cultures? This slide just illustrates peroxisome proliferation in intact liver, determined as palmitoyl-CoA oxidation activity. Again, in untreated rat and mice, there's about similar levels of this activity, and it can be induced with TCA and DCA and the model peroxisome proliferator.

In human liver, the activity is comparable to that seen in the rat and the mouse; however, it is on the low end of the range. However, there is no--it is unclear whether this activity can be induced.

To assay peroxisome proliferation in our cultured cells, we took the cultured cells, treated them for 72 hours with the test compound, harvested them and then the palmitoyl-CoA oxidation in the cell homogenates. And what we observed initially in the untreated cells was that there was a large drop in the basal rate of this activity. So these cells have been in culture for 96 hours now. However, there was an induction with both DCA and TCA as others have shown and we have reported in an earlier paper and with the model compound.

What about human hepatocytes? Human hepatocytes also lost this activity, and the drop was so profound that we could not detect it. It was below our limits in detection. And then, when the cells were given the TCA or DCA or even the Wyeth compound, we could not stimulate it above our limits of detection. So this activity was unusable in our human hepatocytes.

So are there other peroxisome proliferator responses that can be used? One response that has been reported in vivo is induction of cytochrome

P450 4A. This is a microsomal enzyme rather than a peroxisomal protein, and it is involved in the omega oxidation of fatty acids. It is inducible by peroxisome proliferators in rat and mouse liver.

So we asked can we detect this protein in our human hepatocytes, and can we detect induction of this protein? So we used a commercially available antibody to human P450 4A11. Using this antibody, we examined the levels in hepatocyte preparations from three different human livers, and we did observe detectable levels of P450 4A in all of the liver--the hepatocyte preparations. The induction, however, was variable. Human liver six was not very responsive to either the model peroxisome proliferator or to TCA and DCA, although there may be a slight induction with Wyeth and DCA.

The human liver seven and human liver eight cells were much more responsive. We saw a nice enhancement of this protein with the Wyeth compound. However, with DCA and TCA, they seemed to be differentially responsive in that there was a very robust response with DCA in the HL7 cells;

however, there was no response in the HL8 cells, but there appeared to be a minimal response with TCA.

So while you can see induction, the response among different human livers is quite variable, both to the overall responsiveness and to which compounds actually induce the response.

So back to the question can we use hepatocyte cultures to study these peroxisome proliferative effects? In rat and mouse hepatocyte, yes, we can use palmitoyl-CoA oxidation as an index of peroxisome proliferation, and it is inducible with both TCA and DCA. In human hepatocytes, however, palmitoyl-CoA oxidation was not a useful index of peroxisome proliferation. However, we observed cytochrome P450 4A protein and induction of this protein; however, the extent of induction and whether induction occurred were quite variable in different human hepatocyte preparations.

So it is well-known that rat and human cells are different in their responses to the peroxisome proliferator-type compounds, so can we

use the hepatocyte cultures to try to determine why?

As a brief background, messenger RNA levels of the peroxisome proliferator activator receptor alpha are about tenfold higher in mouse liver than in human liver, and there is evidence for some polymorphism among human PPAR alpha. And in fact, in humans taking fibric acid drugs for hyperlipidemia, there is no evidence for increased peroxisomal enzyme activity, although these drugs do induce peroxisome proliferation in rats and mice.

So can we use the human hepatocytes, then, to ask the questions, are the differences in response due to differences in levels of the receptor or differences in the overall activation of the response element, the peroxisome proliferator response element?

First, the levels of the receptor protein itself: we used an antibody commercially available specific for PPAR alpha, it does not crossreact with gamma, to determine levels of this protein in

our human liver preparations and in cell cultures, and what we observed was that the protein is detectable in human liver, although the levels are quite variable.

In fact, human liver two here had levels that were not that different from what you would observe in a mouse. And this protein, the levels of this protein, were maintained when the cells were passaged in culture.

What about the activation of the response element? To do this, we transfected our human hepatocytes with either blank DNA or the mouse PPAR alpha protein along with its partner, the retinoic acid X receptor alpha, and we looked for activation of an artificial response element hooked to chloramphenicol transferase.

In the cells transfected with the blank DNA, there was a very low basal activation of this response element, and we could not detect any significant increase in activation when the cells were treated with the Wyeth compound or TCA or DCA. However, when the cells were transfected with the

mouse protein, there was a large increase in the basal levels of the response element activation, suggesting that there is an endogenous activator in the cells.

And when the cells were treated with the Wyeth compound or TCA or DCA, there was a further enhancement of this activation. So the human cells are capable of responding to peroxisome proliferator receptor activators when you provide them with a mouse protein.

So where do we stand? Can we use the human hepatocytes to study these early events, or can we use rat, mouse or human hepatocytes to study these events? For the mitogenic effect, rodent and human hepatocytes did not respond, as is seen in vivo, so they are not real useful for studying direct mitogenic effects. However, there was an interesting interaction between DCA and EGF that we observed in the rat hepatocytes which we want to investigate further.

As for the peroxisome proliferative effect, palmitoyl-CoA oxidation activity was not a

useful endpoint in human hepatocytes, but it is very reproducible and detectable in the rat and mouse hepatocytes. However, P450 4A induction occurred both in rodent and human hepatocytes and could be used to quantitate differences or to assess differences in human versus rat or mouse responses.

So I just wanted to acknowledge the people who worked, did this work: Jennie Walgren did this work as her dissertation research, and David Kurtz does a lot of the molecular biology work; various technicians and also the support of a DOE cooperative agreement.

Thank you.

[Applause.]

DR. PREUSS: Questions?

[No response.]

DR. PREUSS: Before, when I opened the session this morning, I forgot to thank all of you for showing up this early. Thank you.

Oh, please.

QUESTION: Are you intending to extend

these studies in guinea pig hepatocytes?

DR. MCMILLAN: We don't immediately plan to do that. I know Ruth Roberts has done some work out in Xeneca with the guinea pig hepatocytes, but we haven't planned to do any work with the guinea pig. We want to concentrate with the human hepatocytes.

QUESTION: Stott, Dow. The mouse data obviously didn't work out so well. Is this just an isolation issue, or do you have some other explanation?

DR. MCMILLAN: We don't know what it is due to, and in fact, other people--we've talked with other people, and they've reported the same effect. And I don't know if it's the way we isolate the cells or if there is just something in the mice themselves that makes them hyperreactive when they go into culture.

In fact, back in 1997, when we first started this work, we didn't have this problem. And then, suddenly, something changed, and now, we can't decrease the background levels.

QUESTION: Because we are aware of data being generated in other laboratories where they are seeing response in the mice to TCA and are able to get a pretty robust response.

DR. MCMILLAN: Right.

QUESTION: Trying to put you in touch.

DR. MCMILLAN: Thanks.

MS. SCHALK: Anyone else?

QUESTION: Hello, my name is Jennifer Sass. I'm with the National Resources Defense Council. I had a few questions.

First of all, in your liver cultures, your human liver cultures, were they isolated hepatocytes or were they whole liver cultures? Did they include all of the liver cells? Did they have Kupffer cells in them, or were they monocellular cultures?

DR. MCMILLAN: They were hepatocyte cultures, and they are usually about 95 percent plus hepatocytes with noncontaminating--minimal contamination from either Kupffer cells or endothelial cells.

QUESTION: Okay; so, I'm a bit concerned, I guess, in terms of extrapolating to make broad statements about human liver for two reasons: one is that the cultures that you used didn't represent an intact liver, and that's, I think, important for this context because we do know that Kupffer cells actually are engaged in the human response to peroxisome proliferators. And we also know that humans do respond to the clofibrate-type drugs as well as rodents.

The other thing is I was very interested in the different, I guess--I think you showed it with the P450s but the different kinds of responses you got from your three different livers, isolated from three different humans. So I wondered, in that sense, given already the difference in only looking at I guess under 10, because you had them numbered at least till seven, how confident you felt making a broad statement about humans based on that.

DR. MCMILLAN: Well, I think it suggests that humans may respond very differently and--

QUESTION: Differently from rodents or from differently from each other?

DR. MCMILLAN: Differently from each other.

QUESTION: That's what I was getting at. I mean, to me, what your data looks like is that it is hard to make a broad statement about humans in general from the differences that you have shown one to the other and the fact that the cultures were monocellular cultures.

DR. MCMILLAN: Right; so, you know, the human population is very diverse versus the rat and the mouse cells, which is a very homogeneous population.

QUESTION: Thank you.

QUESTION: Paul Deergard, HSIA.

I'd like to expand a little bit on what Bill Stott was talking about. We have a project at the moment which is directed toward perchlorethylene, where we are less concerned about DCA, in fact, not concerned about DCA, but we are looking at TCA. So what we are trying to do is to

string this thing through quantitatively. So we have looked at the level of TCA generated in the B6 mouse in the inhalation setting; then, looked at the TCA levels in the in vivo setting through drinking water mode and then moving into the in vitro mode, where we can look at the mouse and man.

And we do, as we go across this sequence, we are able to say that the peroxisome proliferation response in the mouse in the in vitro setting are the same level as TCA is generated in the in vivo setting match one another very precisely. We are also looking at cell proliferation through thymidine incorporation, and we do get a response in vitro. The fold of increase is less than it is in vivo, but we do manage to see that response in vitro. So the human work is in progress at this time.

DR. MCMILLAN: I'll have to talk to you about your mouse cells and see how you --

QUESTION: I'm not involved in the detail, but certainly, they are getting the proliferation to occur successfully in vitro, and there are at

least two other labs where that is achieved successfully.

DR. PREUSS: Thank you.

Our next paper will be on TCA and T-cell activation and will be presented by Neil Pumford of the University of Arkansas at Fayetteville.

DR. PUMFORD: I would like to thank the organizers for inviting me to present some of my data on trichloroethylene and autoimmune disease. Specifically today, I will be talking primarily about trichloroethylene and T-cell activation.

Autoimmunity is the loss of self tolerance. It is where your own immune system mounts a response directed against your own cells or your own body. Now, the etiology of autoimmune disease is multifactorial, but it includes a genetic component and an environmental component. And the environmental component consists of possible effects from chemicals.

There are over 80 different types of autoimmune diseases. They range from very organ-specific diseases such as Hasimoto's thyroiditis to

general overall systemic diseases such as s ystemic lupus erythmatosis and systemic sclerosis.

Now, if add all these different autoimmune diseases together as one category, you would find that over 9 million Americans are affected by autoimmune disease; that means one in every five Americans has an autoimmune disease [sic]. That's a major public health concern.

Now, how can a chemical such as trichloroethylene interact with the immune system to accelerate immune response or exacerbate an immune response? And if it does, what are some of the mechanisms or possible mechanisms that can cause this? Now, there's over 100 different case reports implicating trichloroethylene in autoimmune disease, primarily in a systemic sclerosis type of response or a lupus type of autoimmune disease.

I'm going to highlight a couple of different studies that have implied that trichloroethylene is involved in autoimmune disease. Byers et al in 1988 showed that there was alteration in T-lymphocytes and an increase in

antinuclear antibodies or autoantibodies. Kilburn and Washon in Arizona found that there was an association with antinuclear antibodies and systemic lupus. In 1994, Clark et al found that perceived exposure to solvents including trichloroethylene and an increase in antinuclear antibodies.

Now, Nietert in South Carolina showed that occupational exposure based on an exposure, particular job association, found that there was an increase in risk of systemic sclerosis in the males. In the study presented yesterday by Dr. Lacey, the researchers found there was an association with systemic sclerosis, but it was not significant. Studies in mouse, in an animal model, the animal model is, these autoimmune prone mice MRL++ were found by Khan et al to provide an animal model that showed an increase in an immune response in these animals following treatment with trichloroethylene.

So we are using the same animal model treating in the drinking water with

trichloroethylene to try to ascertain what kind of cytokines would produce these autoimmune markers that are and produce nephrotoxicity present in different organ systems including the liver, the lungs and the kidney.

Our treatment is trichloroethylene at the 0.1, 0.5, and 2.5 milligrams per kilogram. This calculates to be for the 0.1 dose and treatment it would be--the 0.5 is around 100 milligrams per kilogram per day. There appears to be a decrease in the 2.5. Now, how are the T-cells activated? We initially used two different markers, CD 44, which is just a marker for T-cell activation; an increase in this T-cell surface marker would indicate activation of the T-cells.

We also utilized another T-cell marker, CD45RB. A decrease in this T-cell surface marker would be indicative of T-cell activation.

This is flow cytometry, gated on CD-4-positive T-cells. This first column is CD-44, and this column is CD45RB. What you would be looking for with CD44 would be an increase or an increase

or CD44 high following treatment with trichloroethylene. As you can see, there is a shift to the right where an increase in the T-cells that are expressing this on the cell surface. Now, with CD45RB, you are looking for a shift to the left or a decrease following TCE treatment.

There does appear to be activation of the T-cells, and it appears to be in a dose-dependent manner in both the CD44 high and the CD45RB low. So we do have T-cell activation; now, what kind of activation is this? The T-cells can mature into two different kinds of mature T-cells, either an inflammatory type of response or a TH1 type of response or a humoral type of response or an antibody-producing type of response or a TH2 type of response.

We use the markers gamma interferon and IL-4 as our markers for these cytokine profiles. SO following TCE treatment, we have a dose-dependent increase and a significant increase at the 0.5 milligrams per mil level of gamma interferon. This is at the 4-week time period

following treatment with trichloroethylene, and we found that there were no change in the IO4 levels following trichloroethylene treatment.

So this is indicative of a TH1 type of response or an inflammatory response. And this same pattern was followed at 32 weeks following treatment with trichloroethylene. There was an increase in gamma interferon with relatively level cytokine secretion of IO4, so it's a TH1 type of response.

We investigated the liver, the lungs, the kidney, for serum markers of toxicity and also histologically for any kind of toxicity or increase in fibrosis. The only thing we found in serum markers was a mild increase in alanine amino transferase levels, which indicates a mild toxicity in the liver.

Now, looking at the liver histologically, we found that there appears to be a massive infiltration of mononuclear cells that were in the trichloroethylene treated group and not in the control group. The infiltration, as you can see

here, was primarily localized around the periportal region and not the centrilovular region.

A pathologist working with us, Dr. Laura Lance, blindly evaluated the histology slides for portal infiltration and found that there was a significant increase at the 0.5 milligrams per mil level, so there was a significant increase in portal infiltration. She also scored the hepatocytes for reactive changes, meaning changes in multiple nuclei, changes in the nuclear, changes in the pathological--in the nuclear picture, and nuclei that were in mitosis.

And in these reactive changes, even at the 21 milligram per kilogram per day level, she found significant differences in the hepatocytes. The picture with a mild hepatic damage, periportal infiltration, and reactive changes, she determined that this picture, the histopathological picture, resembled idiopathic autoimmune hepatitis.

So, not only does trichloroethylene cause an increase in an autoimmune response, such as increases in antinuclear antibodies, it also causes

or exacerbates an autoimmune hepatitis.

This is our overall scheme for possible mechanisms by which trichloroethylene could cause an autoimmune disease. You know, of course, there is polyrheummetabolism; there is covalent binding of proteins. There is activation of T-cells into a TH-1 type of response. D cells must be involved, because there are increases in antinuclear antibodies.

We also found increases in an antichemical response, but we find that that's probably a minor response. Endothelial cells are damaged by increases in--there's an endothelial cell injury. There's activation of macrophages by proxy nitrate and nitration of tirosines. But we want to focus on what could be--is one of the metabolites. Is a metabolism required for this activation of T-cells?

So in this next study, we used the same mice, the MRL++ mice and treated it for four weeks with the trichloroethylene. We also had an osmotic pump that chronically treated the mice with diallele sulfide. And in this study, this is a

Western Blot with antibody events; the cytochrome P450 2E1. Diallele sulfide is a cytochrome P 450 2E1 inhibitor, so we were trying to inhibit the metabolism.

You can see in controls there's a normal high level of the CYP2E1. With diallele sulfide, this is dramatically decreased, and with th e TCE, it was also decreased, and TCE with diallele sulfide was an additional decrease.

Now, looking at metabolism, utilizing the antibody against the modified proteins, with trichloroethylene, there was the normal 50 kilodalton protein. This is just an indication that there is oxidative metabolism by cytochrome P450. And when you inhibit the CYP2E1, there was a decrease in metabolic activation, so there's a decrease in oxidative metabolism.

Now, is there some sort of decrease with a marker for T-cell activation? This is using mice treated with trichloroethylene or diallele sulfide; the mitogen response to Con A. With just TCE, there was a dramatic increase in the mitogen

response, so there was an increase in activation, and this was blocked by inhibiting cytochrome P450 2E1, so metabolism is required for the T-cell activation.

Now what could be some possible mechanisms by which trichloroethylene or its metabolite, primarily its metabolite, could interact with the immune system to cause this T-cell activation? It's an overall T-cell activation, not just a response directly against a certain protein. So in order for that to happen, there has to be overall panactivation of the T-cells, and that can happen, say, with superantigen; it could activate T-cells in the nonspecific pan activation, or it could be an increase in hytolonic acid, which is the receptor for the CD44.

But there recently was a paper produced by Rhodes et al; it's a Nature paper, and by--the normal activation of T-cells is through a T-cell receptor MSE class 2 with a presentation of peptide. This is signal one. But you also have costimulatory molecules such as B7 and CD28. The

interaction of B7 and CD28 is a shift-based formation, and this initiates the signal transduction to activate the T-cells.

Well, Dr. Rhodes has shown that you can use an aldehyde in place of the B7 that will form a Schiff's base space on the CD28, and that alone can activate the T-cells in a nonspecific manner. So our working hypothesis is that--thank you--trichloroethylene is oxidating metabolites to trichloroacidaldehyde. This acid aldehyde can interact with the CD28, forming a Schiff's base, and of course, trichloroacidaldehyde chloral is in equilibrium with trichloroacidaldehyde hydrate or chloral hydrate.

So today, I'm going to refer to this as trichloroacetaldehyde hydrate just to emphasize the this is an acetaldehyde capable of forming a Schiff's base with CD28. Now, first of all, can trichloroacetaldehyde form a Schiff's base on the cell surface of T-cells? Using flow cytometry and using the antibody that was produced to dichloroacetyl chloride, an adduct, we found that

that cross reacts, that antibody cross-reacts with the Schiff's base. The Schiff's base is also stabilized with sodium cyanoborohydrate so that we can detect it with the flow cytometry.

This is an increasing concentration of trichloroacetaldehyde hydrate incubated on T-cells, and you can see there's a concentration-dependent increase in Schiff's base formation on the T-cells. We also found--this is a proliferation assay in which you had a very low concentration of anti-CD3, high concentrations of anti-CD3 will cause proliferation in T-cells. But at low concentrations, you can see an effect from trichloroacetaldehyde hydrate at 0.2 millimolar and 1 millimolar concentrations. So the aldehyde by itself can activate T-cells or cause an increase in proliferation.

We also looked at, on the cell surface, CD28, a cell surface marker, is not only the costimulatory molecule but it is also a marker for T-cell activation. On this side, we didn't treat the cells with anti-CD3. You can see by itself,

the trichloroacetaldehyde hydrate increases the T-cell marker for activation. With low levels of anti-CD3, this increase is dramatically demonstrated following TCAH incubation.

Another marker for T-cell activation, which appears to be a very sensitive early marker, is the increase in CD-62L, I mean, the decrease in this CD-62L, so you're looking for CD-62 low or a shift in the curve to the left. And following incubation with TCAH, we found a dose-dependent increase in this activation marker of CD-62L, a decrease or increase in the low.

So T-cells are activated just by incubation in vitro with the aldehyde. Now, can the same thing be produced in vivo, so in our same animal model, the MRL++ mice, they were treated in drinking water with trichloroacetaldehyde hydrate, and we looked at T-cell activation markers. We found that there was a dose-dependent increase in the T-cell activation marker CD-62 L low, so the T-cells following treatment in vivo with trichloroacetaldehyde hydrate activated the T-cells, and

they activated it in a TH-1 type response with increase in gamma interferon in a dose-dependent manner with IL-4 remaining stable.

I'd like to thank primarily Kathleen Gilbert, who is the immunologist that's working on the project and my postdoc, Sara Blossom. Joe Griffin did the initial TCE in the MRL++ mice and Dr. Laura Lamps for our histopathologist. And I would like to entertain questions.

[Applause.]

QUESTION: Hi, Jay Pandey from Medical University in Charleston.

I was interested in your costimulatory studies with B7 and CD28. Did you get a chance to check out the competitive antagonist of CD28, that is, CTLA4?

DR. PUMFORD: Have we antagonized it? No.

QUESTION: So you don't have any data on the CTLA4, which is the negative revelator of the costimulation.

DR. PUMFORD: I'm confused now.

QUESTION: CD-28 and B7 should be there.

Now, there is a competitive antagonist of CD-28, CTLA4. Do you have any data on TCE and CTLA4?

DR. PUMFORD: No.

QUESTION: Now, one other question.

DR. PUMFORD: We don't even know for sure that the aldehyde is binding to that area yet.

QUESTION: Okay.

DR. PUMFORD: Because we haven't blocked it. We haven't shown it binding directly to that. We've found that it is binding to the cell surface. We don't know where yet.

QUESTION: In humans, autoimmune chronic hepatitis is SLA associated, and my question is, does the H2 genotype occur in mice, it is analogous to or homologous to the HLA mice you used?

DR. PUMFORD: Yes, I know. I'm sorry; I don't remember. Kathleen Gilbert would know that.

QUESTION: And you did not find any fibrosis, no effect on fibrosis?

DR. PUMFORD: No effect on fibrosis. That's one thing I meant to point out is that in our model, we did not find the fibrosis, which is

the classical indicator in systemic sclerosis, which is primary disease that you found.

QUESTION: How did you check for it? Did you do a collagen assay?

DR. PUMFORD: Both, histologically and markers.

QUESTION: Dick Bull, MoBull Consulting.

Just to help my ignorance, what's the basis of the sensitivity of the MRL++?

DR. PUMFORD: Why are they sensitive?

QUESTION: Yes.

DR. PUMFORD: The MRLPR have a defect in their liver LPR gene, so they develop lupus very early in their life. But the MRL++ mice, they're not sure exactly why, but they are genetically predisposed to, within the first two years of their life, they will develop lupus-like symptoms, the mice, and usually die within two years due to kidney failure.

QUESTION: Joann Caldwell, USEPA. I was looking at probably about your third slide, when you, I believe, antibodies, autoantibodies, and it

looked like you had a paracentral increase in staining in your hepatocytes. And yet, later on, when you did other experiments, you had a periportal infiltrate.

I wanted to see if you could comment on that, and also, did you see evidence of Kupffer cell activation in the periportal triad with your infiltrates?

DR. PUMFORD: Those are very excellent questions, because that's what I pointed out in the manuscript. The adduct formation on the metabolism was centroovular. The disease is periportal. So how does--you know--why is there a response against the periportal? I do not know the answer to that, but I do, now that there is--

QUESTION: That tends to be where the Kupffer cells hang out.

DR. PUMFORD: Histologically, we looked for Kupffer cell activation using, you know, nitrotirosine as a marker for activation of the Kupffer cells, and it appears to be throughout the whole liver. And they are activated. But it was

not centroovular, not periportal. It was panglomular, to answer the second part.

QUESTION: Larry Moore, Medical University of South Carolina. I was wondering with respect to the infiltrate of the liver, whether you looked at the CD4/CD8 ratio, assuming that there are nomoceylymphocytes.

DR. PUMFORD: We attempted to do that using immunohistochemistry, and the antibodies were human and did not crossreact with our mouse. All we were able to do was detect CD3, so we know it's a mixture, you know, we know it's CD3 positive; in fact, not all of them are CD3 positive, so we don't know the ratio of CD4. We did try to do that and were unsuccessful

QUESTION: And I am also interested in whether you saw or looked at some of the same markers that we typically see in human autoimmune hepatitis where, you know, hypergammaglobulin anemia, you know, monoclonal hypergammaglobulin anemia is common; certainly, antinuclear antibodies, ANCA, those type of things. Did you --

DR. PUMFORD: We found increases in immunoglobulins.

QUESTION: Right.

DR. PUMFORD: Hypergammaglobulin anemia. Other markers, we did not look at. But we did find an increase in immunoglobulins, both ITM and ITG and think it was primary--I forget.

QUESTION: Thank you.

DR. BURCH: Jim Burch from Colorado State. I was--if I understand and followed all of this correctly, you are hypothesizing that the Schiff base protein adduct of TCAH that forms with CD28 is the adduct that is stimulating an autoimmune response, so I was just wondering, did you do the experiment to look for autoantibody formation after exposure to TCAH?

DR. PUMFORD: Yes, and this is very preliminary data that data we looked at it so far at the four weeks, we did not see it. But that's not unusual. It could happen at the 16 or the 32. This experiment will go out 40 weeks, and it has not been done yet.

DR. WANG: Jung-Der Wang from National Taiwan University. My question is when you say that you have not found fibrosis in the liver, how long have you treated these mice?

DR. PUMFORD: That group of animals were treated to 32 weeks.

DR. WANG: Could you give me an idea, these 32 weeks are comparable to human, how long, in life span?

DR. PUMFORD: The mouse won't live much more than 3 years at the most, so 32 weeks is middle-aged, I guess. But you have a good point. We are extending our experiment to try to see if there is fibrosis later. That is one of the things I am hoping for that we will have to see.

QUESTION: Miles Okino with the EPA. I was wondering if you looked for IL10 or TBF-beta or other markers that are somewhat associated with what people are calling regulatory T-cells.

DR. PUMFORD: We did not. Those are good suggestions to do that, but we have not.

QUESTION: Do you see autoantibodies to

the adduct that you're demonstrating? I'm not talking about the chloral hydrate adduct, but I understand that to be a dichloroacetyl--

DR. PUMFORD: We find antibodies against it?

QUESTION: Yes.

DR. PUMFORD: Yes.

QUESTION: So you do get some autoantibodies there?

DR. PUMFORD: We have autoantibodies against proteins that are modified by the drug, by the chemical.

QUESTION: Right

DR. PUMFORD: But it's not a very impressive response.

QUESTION: Okay.

DR. PUMFORD: So I don't think that's a major contributor to the disease.

QUESTION: John DiSesso from Mitre Tech Systems. What disease are you proposing that this is associated with?

DR. PUMFORD: Is trichloroethylene

associated with in my animals? It's autoimmune hepatitis. Is that the same as it is in human exposure? Generally, human exposure seems to be associated with systemic sclerosis.

QUESTION: Is there an animal model?

DR. PUMFORD: But there's always a component of both lupus and systemic sclerosis of an autoimmune component to the liver similar to autoimmune hepatitis in humans in the idiopathic disease.

QUESTION: So if you extrapolate that, do you think that then TCE exposure can be associated with some of these, like, you know, sclerosis or with SLE?

DR. PUMFORD: I'm not saying I know it is or anything. This is what we get in our animal model, and the epidemiology is--

QUESTION: How big were your group sizes? You had graphs that had arrow bars, but how many animals were in this? You said it was preliminary data. How preliminary?

DR. PUMFORD: The last ones were

preliminary. The data before that it was not preliminary. It was eight animals per group.

QUESTION: Okay; thank you.

DR. PREUSS: Good; I think we have to move on. Thank you for all of the questions. Thank you.

And our next speaker is Paula Johnson from the University of Arizona-Tucson, and she will talk about TCE and fetal heart development.

DR. JOHNSON: All right; I'd like to thank Kate for her assistance and the EPA for inviting me to come and participate. They've asked me to come and talk about our research with TCE and fetal heart development, so I'll give you a little bit of history first.

We started looking at TCE based on some epidemiologic studies that were done by Dr. Goldberg, who is a pediatric cardiologist that I worked with. He noticed that there were a lot of his patients coming from one area, two basic ZIP Codes in the Tucson basin, that had a high incidence of heart defects compared to the rest of the children

that he was seeing in the Tucson area.

We all know it's a worldwide contaminant. In Tucson, back in the fifties, we had a lot of airline or airplane industry going on. They had used TCE as a degreaser or solvent; clean the engines off and taken the waste way off into the desert, out away from everybody, long ways away, dumped it into the desert, and by the 1980s, that TCE had gone into the groundwater and traveled toward the City of Tucson.

The wells were closed in 1980 because of high levels. He (Dr. Goldberg) noticed that this happened to be the same, the plume of contamination was the same as the ZIP Code that he was seeing these high numbers of patients from, so they did an epidemiologic study and found that indeed there was a difference between those patients; there was about a threefold increase in that area compared to the rest of the Tucson basin. So that's why we started in this project.

Initially, we looked at the chick embryos just to see if there was in fact something going on

between TCE, DCE. There was also chromium involved, but we haven't examined that aspect to date.

So, we looked at the chick embryo and used TCE and DCE at high levels and found that indeed there were an increased number of heart defects and a variety of heart defects, which is just what we found with the human patients.

The next step was to look at the mammalian model, and we chose the Sprague-Dawley rat because it demonstrated a low incidence of spontaneous heart malformations, same as with the human population, about 2 to 3 percent. We initially did an intrauterine study, which was a pretty provocative way of exposing these animals to TCE, but we wanted to know if this was actually going to be a problem. If we couldn't demonstrate heart defects using that type of a model, there was no point in going on.

So obviously, we found an increased number of heart defects and decided to use a model that was more similar to how humans were being exposed

in the drinking water. We looked at both prepregnancy or that time before they became pregnant, before pregnancy and during pregnancy and then during pregnancy alone. We used high doses of TCE; 1100 parts per million was our max dose.

We did find that there was a significant increase in the number of heart defects that were found if the animals were treated during pregnancy. If it was just before pregnancy, we didn't get an increased number of defects. So it looks like it is occurring during organogenesis.

We found a significant increase, and again, these were at the high levels, 1,100 parts per million, and a variety of heart defects was found. So the next step was to do a dose-response study. We used the same methodologies that we used in the prior studies. We monitored the females on a daily basis. We gathered time pregnant animals, so we knew exactly when their pregnancies began. And on that first day of pregnancy was when we started the TCE drinking water.

The waters were changed every 24 hours, given a fresh solution. There was about a 35 percent degradation of TCE, so that was taken into account. We made special water bottle covers to decrease a light breakdown and then changed them every 24 hours. They were exposed during their entire pregnancy, so from day 0 through day 22. The females were weighed every day. Their health was monitored. We wanted to make sure that this was not affecting the pregnancy in any way. We didn't have any results that were affected by the females and their pregnancies.

We then would examine, at day 22, and the animals were euthanized. We looked at the maternal rat for any abnormalities; we looked at the fetuses for abnormalities; looked at their hearts in situ and then removed the hearts for later examination.

There were three of us looking at the hearts. We had Dr. Goldberg, a pediatric cardiologist, Dr. Dawson, who was a pathologist, and myself as a veterinarian, looking at these hearts individually. If we felt they were

abnormal, they were placed into another pile, and we would all go through them together.

To be on the conservative side, all three had to agree that yes, that was a defective heart. If there was any question, it was placed in the normal batch, because we wanted to be on the conservative side.

In this--the concentration levels we used in the dose-response study, we used the high dose of 1100 parts per million, which was the maximum solubility we could get; about a hundred fold lower dose of 1.5 parts per million. We used 250 parts per billion based on the fact that the high level found in the Tucson wells was 270 parts per billion, so that was our low point. Then, we went hundred fold lower at 2.5 parts per billion.

I also put on this slide the average dose on a milligram per kilogram per day basis. This was taking into account the breakdown of the TCE over that 24 hour period; the amount of water that the animals drank, that was recorded every day, in association with their weight gain, which did not

stay the same. As they were getting pregnant, that increased. So it was a fun mathematical chore trying to figure all that out. So these are the doses, the average doses per kilogram that we used.

Now the groups the we used, we needed statistically 100 fetuses per group in order to gain statistical significance. So the number of maternal rats varied depending on the number of fetuses that they had. We have a larger number of control animals, because we ran concurrent controls with those groups.

The types of heart malformations that we found were varied, just as has happened from the beginning in the human studies or the human epidemiology study, the avian study and then the rats. There is a large variety of heart defects. The primary ones that we found were ASDs, atrial septal defects, and VSDs, ventricular septal defects. This makes it difficult in trying to figure out, at a molecular level, what's going on, because all of these things occur in the whole span of heart development, so it makes it very difficult

to pinpoint what TCE is actually doing and how it's perturbing the genes.

We are using the atrial septal defects and the VSDs to kind of pinpoint and give us a starting place as to where to begin, so we'll get into that in a minute.

The number of abnormal hearts that we've found, you can see on the far right, the 1,100 parts per million showed a statistical significance looking at on a per fetus basis. Now, the two lower levels, the 1.5 parts per million and 250 parts per billion, were about double that compared to the control, but they were not statistically significant. And we don't know why, but the 2.5 parts per billion showed no heart defects at all.

Now on a percent litter of abnormal hearts, this meant that in a litter, they had to have at least one abnormal heart, and at the high dose, we had 66.7 percent abnormal hearts, and again, the two lower doses, they were increased, more than doubled from the control, but not statistically significant.

We then gave all of our data to a statistician, and she did a probit analysis to look at the expected effective dose, to see if we have actually a dose-response going on. And on the lower corner of the left--sorry--from here to here is where our studies occurred, but carrying this up with the probit analysis, the effective dose of 50 percent animals that would have had heart defects would have to have 2,692 parts per million.

So it's a high level, but this gives us an indication that there is in fact a dose-response going on with TCE.

The other study that I was asked to talk about today was comparing our study to one that I was involved with with Dr. Fisher and his group at Wright Patterson. They asked me to get involved so that we could be using the same method of looking at the heart defects, so we were having the same SOP, basically, for examining.

Their study used rats. They did a gavage of TCE on a daily basis from day 6 to day 15, and the differences between these two studies have

raised a lot of questions. They only found 4.5 percent or we in that study only found 4.5 of the fetuses with abnormal hearts, whereas our study, using the drinking water, had 10.4 percent. That's a pretty big difference.

They had 60 percent of the litters with malformations, whereas, we had 67. Our controls were pretty close, 2.9, which is in that 2 to 3 percent range, and they also used a soybean control at 6.5 percent.

So why did we have these differences? What was different about these two studies? In thinking back on them and trying to figure out what is going on, we feel that it's probably the method of delivery, difference between a soybean oil gavage versus the drinking water, which is on a 24-hour basis, and the days that they were treated. In the Wright Patterson study, they used gestation day of day 6 to 15. The heart has already started to differentiate by day 6, so we may have missed some of those early defects that would have gone on had it been treated earlier, and in the drinking

water study, it started from day 0. So it has raised some questions and some things that we need to look at.

The next portion of our studies, which is what we're currently working on, is the genetic expression, how is TCE affecting the heart to cause these defects? We are again using timed pregnant dams, and they start their treatments on day 0 of pregnancy. We are collecting the embryonic hearts at day 11. This is a time when there is a lot of developmental processes specifically with the heart that are going on. That AV canal is starting to form. The heart is beginning to loop. So there is a lot going on in developmental times.

To do it earlier would be good, but there's not enough tissue to generate for the RNA analysis that's needed. So this was the best time point. There is a lot of data that has gone on in the mouse for this time point, so we know genetically what is happening and what should be expected.

So we used RNA isolation, collected the

RNA; did subtractive hybridization and then used several assays. We generated 160 clones to analyze, and these were grouped because there were so many based on their functionality, so we had housekeeping genes, stress response genes and then the potential developmental processes dealing with the heart and the cardiac development. And we had 9 CDNAs that were sequenced that showed to be sensitive to TCE exposure.

So the two that we kind of homed in on based on some other studies and with the mouse models was the Serca 2A, CA2+, ATPase, and the rat GPI-p137 genes. As you can see on the right, the expression of those genes is decreasing. It's being downregulated as the concentration of TCE increases. And the control is on the left; 100 parts per billion up to 100 parts per million, and it does show a dose-response, as we found with the live studies.

So the conclusions that we've come to is that TCE exposure in rat does cause an increase in cardiac malformations when given in the drinking

water exposure, and that's started from the beginning of pregnancy to the end. It does seem to indicate a dose-response based on our live model and on the embryonic tissue. The downregulation of the genes, Serca 2A and P137.

So the goals of this project are to continue with the gene sequencing and see if we can more delineate the molecular mechanisms of how TCE is involved in causing these heart defects. We do need to stress that we can't extrapolate directly to humans. That's not quite possible yet. We're working on it. We obviously feel this is important, and there is a link, and that's why we're continuing.

These are the investigators that I've worked with over the years. Dr. Dawson and Dr. Goldberg were the two who were involved at the beginning. They have both since retired. Dr. Selmin is the molecular biologist that I am currently working with, and she's doing a great job looking at the molecular aspects. Dr. Collier is a graduate student who has started working with

these, and he is now over in California. Dr. Mays is our statistician, who without her, there is no way we could do any of this.

And then, the research technicians have been fantastic. Everyone involved in this project has been very dedicated, and we all believe in what we're doing, so it's been a good project. And we really need to thank the NIEHS for their Superfund grant support. It's kept us going.

Answer any questions?

[Applause.]

QUESTION: Hi, I'm John DiSesso from Mitre Tech Systems.

It's an interesting situation. One of the things that I notice about your data--I've looked at your data a number of times--is that there's certain statements that you make that I don't know where they come from, for instance, that the heart is differentiating on gestational day six. When you say differentiating, what do you mean by that by that in that sense? Because by day 6, the zygote is just implanting.

DR. JOHNSON: It's just implanting, but cardiac looping is starting to begin; it's already started to--I don't know how to exactly word it.

QUESTION: Well, I would be very interested. I would really like to see the data on that, because I've done a lot of work on that, and there shouldn't be any looping at that point.

DR. JOHNSON: Well, it's the beginning of all of those systems have started. That's why we're doing it at day 11. We're taking the embryos at day 11 because that's when it's starting.

QUESTION: But the claim is that the dose, the difference in when the material was administered, beginning on gestational day 6 or beginning on gestational day 0, the implication is that the exposure, I guess, needs to be occurring earlier than implantation in order to have these effects. And I'm just wondering why you believe that.

DR. JOHNSON: Well the fact that we're getting--we're seeing such a large difference in the number of heart defects from those who are

treated from day 0 throughout pregnancy from those from just day 6 to 15, there has to be some kind of a difference that went on, and whether it's the method of exposure, whether it's just the one day dose versus the continual, or whether it's that onset of when the heart is starting, I mean, that whole process starts before day six. Even though it has not yet implanted, the whole embryo is developing.

So to get those started earlier, we're not sure, and that's why we need to do more research on that.

QUESTION: All right; I'll let you go on that, but I'm not convinced. But one of the other things I wanted to ask you about, then, is when you're looking at these things, your idea that you need 100 fetuses to get statistical significance is interesting, because when one does safety assessment, one isn't worried about the number of fetuses. When you do statistics based on the fetal numbers, you're going to overestimate your risk. So most people do two things. First of all, they

increase the number of litters, and generally, it's acceptable that you have at least 25 per dose group, and I think the highest you had in the treated groups was, like, 13.

DR. JOHNSON: Right.

QUESTION: And that the litter percentage that you used isn't the litter percentage that's normally used. Normally, you take the percentage of affected animals per litter; this is like a percent of a percent. So instead of saying I had 6 out of the 10 litters had an affected dose doesn't mean I had 60 percent affected litters. They look at the number of animals in each litter as a percentage and then average those to get rid of some of the big variance, because you get a lot of variance in that.

And with the small number of animals you have, it's tough to understand where your data are going to take us.

DR. JOHNSON: Well, if, yes, we could get the funding to do a lot more animal studies, we'd do it. It's been a real problem to get the animal

numbers. I mean, we've dealt with the statistician to try to generate the numbers that we needed, and when we first started these studies, people were looking at the number of fetuses, not the number of litters. This was 15 years ago this was started. So we have been trying to keep consistent with that yet increase the numbers.

It's been a juggling nightmare trying to get the funding to do all this. And that was 1,100 hearts to do this dose-response study; that's a long-term project to do, so and it's very time consuming to do that.

QUESTION: Have you done histological things at times during gestation to see if there were actually effects on the heart that you could pick up?

DR. JOHNSON: We did initially. When we first started in 1988, we did histology studies and found that there were no differences. The myocardium does not seem to be affected, at least from the initial studies that we did.

QUESTION: Okay.

DR. PREUSS: Dr. Bull?

DR. BULL: Yes, this is Dick Bull from MoBull Consulting. I'm interested in two things, mainly. Have you published the information on the molecular changes? I don't remember if those were transcripts or--

DR. JOHNSON: No, it's in Birth Defects Research. It was published this last year.

DR. BULL: Last year? Okay; and you're the first author?

DR. JOHNSON: No, I think I'm third on that. Dr. Collier was the first author on that.

DR. BULL: Okay; thanks.

DR. BURCH: Jim Burch at Colorado State. Thanks for an interesting talk. I was just wondering, I wonder about whether there was any aversiveness to consumption of drinking water at those high doses. Did you look for differences in weight gain in the mothers or anything like that?

DR. JOHNSON: We sure did, and we were concerned about that, because the high levels of TCE, I mean, it smells bad; it tastes bad. As a

veterinarian, we don't give anything to animals unless we take it ourselves. So I tried it. It's horrible.

[Laughter.]

DR. JOHNSON: After about the second day, they adjust to the water, and they drink it. We had a couple of instances where the water bottles would break, and we'd have to give them regular tap water. They wouldn't drink the tap water. They like the TCE after they get used to it.

[Laughter.]

DR. JOHNSON: Yes, it was really rather odd. When we did this study with the metabolites, the TC ethanol, they're really very happy rats. They really liked that one. But the TCE and the TCA that we've tested, they do seem to adjust to it, and they drink normal amounts of water. We monitored the water every day. We monitored the weight gain to make sure we were getting adequate amounts of food and water into them, and they do adjust really well.

Well, thank you.

DR. PREUSS: One second, I think there may be one more question.

Paul?

QUESTION: Paul Deergard, HSIA. I should probably point out that it's not just your studies versus Jeff Fisher's studies, but there are a number of other studies, inhalation studies including some very high dose studies done by EPA where there are no reports of increased cardiac abnormalities as well, just to keep the record straight.

DR. JOHNSON: Yes, I was just asked to comment on the fact that I was involved with Dr. Fisher's study as well, so that's why I could comment on that one, but yes, you're right. There are some differences, and that's why we're not exactly sure we're looking at the genetic level to see if we can determine what genes are being perturbed.

QUESTION: Just a quick one. Did you look for any other malformations besides --

DR. JOHNSON: Yes, we did. When we

initially would sacrifice the animals, we looked grossly at the mother and at the fetuses. We found very few other abnormalities. We found no tumors. Birth defects, otherwise, we found a couple. I mean, much lower than we would have had expected on a normal basis, but both the controls and the treated animals were statistically normal

QUESTION: Yes, because something with that response, you would expect to have seen a number of other changes as well.

DR. JOHNSON: Yes, we did; long, short tails, toes, all that; everybody was really normal and normal weights. We expected to see some lower weights, but the fetal weight was fine.

DR. PREUSS: Well, thank you and we thank the first three speakers.

We have a little extra time for our break, but we'll reconvene at 10:00 promptly. Thank you.

[Recess.]

DR. PREUSS: Can I ask everybody to take their seats so that we can get started again, please?

Our next speaker is Dr. Guengerich from Vanderbilt University, and he's going to be talking about metabolism of TCE and covalent binding of reaction products.

DR. GUENGERICH: Good morning. I'd like to talk to you about the indicated title. I appreciate the opportunity to be here. I've been working on trichloroethylene in one way or another off and on for the last 25 years. In the last year, I have been actually involved in a practical issue with alleged trichloroethylene contamination in Dickson, which is about 40 miles away from Nashville, and you can read some of our comments on the issue at our Website for the toxicology center.

There had been quite a bit of press. It turns out we really don't know how much trichloroethylene is there or if that's really a problem or not. Unfortunately, most of the attention has been paid to some potential tort cases, and when some of the celebrity lawyers pulled out, I think because of lack of interest or available money, the press has died down on this

particular subject.

Well, that's so much for the practical issue. Now, I'm going to talk about the science or the basic science, I should say, and I'm going to talk primarily, well, really, everything I'm going to talk about is going to be about basic metabolism and also some raw chemistry. I should apologize. I think in vivo work is very important, but I'm not going to talk about any today here.

I'm going to talk about reactive metabolites and the potential for these being involved in some of the damage. First I'd like to just mention epoxides, and the epoxide of trichloroethylene will be the central object of what I'm going to talk about today. In general, when we have aromatic compounds or particularly olefins like trichloroethylene, we have the potential to form epoxides.

Epoxides have a number of fates. Because of their ring strain, they tend to react with nucleophiles like protein and DNA, and they can also be intercepted by the enzyme epoxide

hydrolase, which gives a diol, or a conjugation with glutathione transferase to give a glutathione conjugate. As far as we know, these processes are not relevant in the case of trichloroethylene, however, although we do get some of the epoxide, and that's been the central object of our interest.

Now, if you read the literature in this area and about epoxides, one gets the idea that perhaps these are magical things that just sort of fry things immediately all the time. That's not really true. They vary considerably in terms of their half-lives, and they also can vary considerably in terms of their biological effects.

So, for example, if we look at this old study here by Norman Drinkwater with Jim and Betty Miller, it turns out that some epoxides are quite stable, such as styrene oxide. And it's not terribly genotoxic; it is genotoxic but not all that much, and in fact, it has a half-life in water of about 5 hours or something like that.

And as we go up to some of the benzopyrene-derived epoxides, they become less stable and more

biologically reactive. Okay; what about things that are of relevance in the way of halogena ted olefins and related compounds? We've made a number of compounds, and some of these are not halogenated olefins. And this comes from urethane, but over the years, I think well this comes from the Millers' work but all of these other compounds have been made in our laboratory, and you can see that they vary in stability too.

These are rather unstable. And in fact, let's go to the focus here. This is the epo xide of trichloroethylene. We have synthesized that. That, if you put that in water, it has a half-life of about 12 seconds. If you go to vinylidine chloride epoxide, that half-life is down around 2 seconds. Just for comparison, the half-life and reactivity don't necessarily predict genotoxicity. It turns out that we've made the epoxide of aflatoxin B1. This only has a half-life of one second. It's extremely genotoxic.

On the other hand, these epoxides don't seem to be very genotoxic. The epoxide of vinyl

chloride has a half life of about 90 seconds, and that's actually pretty genotoxic and mutagenic here. So these are some of the things we've done and gives you some kind of a glimpse into what's going on.

Okay; what about the metabolism? Well, years ago, when we got into this business, the literature seemed to indicate that the epoxide was a very transient precursor of chloral. We showed that that wasn't really true. Our interpretation is that there is an enzyme intermediate formed by the cytochrome P450 enzymes that partitions in two different ways.

One way is to go directly to chloral, and then chloral goes on to form trichloroethanol and trichloroacetic acid, and these can form glucuronides, as you realize. We also have a partitioning of this intermediate into the epoxide. Now, we've made the epoxide, and we've looked very hard, and you really cannot get this to form chloral. So this is not on the pathway.

This epoxide, as I mentioned, has a half life of 12 seconds. We have identified the major products. One of these is dichloroacetic acid, and this was the object of some discussion yesterday. We think this is probably the origin of the dichloroacetic acid. This is a minor product compared to the trichloro compounds up here, and we also get glyoxylic acid, and we get large amounts of these one-carbon products. That is, we are splitting the carbon-carbon bond, forming carbon monoxide and formic acid.

This is of particular interest, and we've wondered about how you're getting the carbon-carbon splitting for some time. Some of our early schemes had proposed formyl chloride as an intermediate on the way, and I'm going to come back to this later. It turns out that both of these proposed mechanisms are wrong, but we do know the answer now.

I'm not going to say much about this. Larry Lash talked about the glutathione dependent conjugation yesterday. There's been a lot written about that, and it probably has relevance in

different systems. But I'm not really going to talk about that.

One also generates an acylchloride and a thioketene there as the reactive intermediates. Here's thioaldehyde as well. I'm not going to say much more about that today. We're going to focus on the oxidative systems. You've already heard that cytochrome P450 2E1 seems to be the major culprit here as far as we know. We've known that for, well, over 10 years now that P450 2E1 is the major player in humans and in animal systems with most of these vinyl monomers and halogenated hydrocarbons, including trichloroethylene, so that's going to be the object of the main focus today.

I should point out--and this is what I think is an important issue in terms of any kind of risk assessment or at least kinetic predictions--there's a variability in the human enzymes, particularly the cytochrome P450s, and this has been mentioned this morning in regard to P450 4A11 in humans. It also happens with P450

2E1, and we've been involved in some efforts over the years in collaborations with PBPK analysis, particularly with methylene chloride, not really with trichloroethylene in this regard.

There are a number of issues that really come up when you're trying to really model populations where you have variability. The variability of P450 2E1 is more than an order of magnitude in the general population. And that becomes important. That's in liver. We don't really have a good grip on this in some of the extrahepatic tissues such as lung, heart, things like that.

There are also some issues about which in vitro parameters one really plugs into some of these models. Do you use Vmax, Km, or the actual catalytic efficiency here? So maybe other people will talk more about that, but it's not trivial in terms of some of these decisions.

There have been some efforts, particularly with drugs, using in vivo clearance in terms of modeling and dealing with this variation in the

population in particular enzymes, introducing uncertainty factors based on that, making a long--I can refer you to these. These particular ones are not with P450 2E1. They're with 2D6 and 3A4. Turns out that these factors of uncertainty increase with the encretion fraction of metabolism due to a single enzyme.

That is, if you have the metabolism spread out among several enzymes, you don't really have so much variation. If everything is due to one enzyme in question, then, you're going to get a lot more uncertainty, and we're going to have wider variations in the populations.

So let's go back and talk about the chemistry. I told you that some of our early hypotheses about how trichloroethylene broke down were wrong, and so, eventually, we did work out what we think is the final answer. I won't go through the details of this. This has been published in JACS a few years ago, but we took the synthetic epoxide, and we did a number of experiments with O18 and deuterium-labeled water

and looked at the final products and their incorporation of deuterium and oxygen 18, and these are really the only pathways that will explain everything.

I want to point out a couple of things here. You get down to these acylchlorides, and that is going to be very important in forming adducts. Here, we have this derivative here, which is an acylchloride; here, we have an acylchloride. These are entities that can potentially react with proteins, and here we have another acylchloride that is going to be important as well.

We went on in that particular study and defined the mechanisms by which the epoxide reacted with nucleophiles or at least with lysine. And this was done the same way. We took the epoxide and, by scheme of going through those labels and finding out by mass spectrometry where they were, here are the products we get and the mechanisms. So you can see that the initial attack is not on the epoxide itself. And in fact, if you start pushing arrows around, you won't get any stable

products out of that anyway.

The intermediate, really, here are these two acylchlorides. This one reacts with the epsilon group on lysine to form this dichloroacetyl derivative, and we also get this glyoxal derivative and also formyl lysine. This turned out to be very important. Why? Because we did more studies with real proteins, and most of these things are done with albumin as a model. You can then digest the protein with a proteinase K and do mass spectrometry, HPLC mass spec, to analyze your products; here's formyl lysine; here's dichloroacetyl lysine.

And it turns out--focus your attention on this box here--that if we react trichloroethylene oxide with albumin, here's the dichloroacetyl lysine adducts, and I think Dr. Pumford had indicated that these were detected with a particular antibody. It turns out that you have an order of magnitude more formyl lysine adducts, so I think if you're quantifying the covalent adducts formed with protein based on this, this is the

proverbial tip of the iceberg.

We really have a lot more of these adducts floating around, and these are reasonably stable, at least stable enough to survive the overnight digestions with the proteases to get to this particular point.

Now, this slide--let me go one more and then I'll come back. I meant to change that. I told you about lysine, but we did another experiment, and I'll try to explain this quickly. We're using insulin here. Insulin is a model here. This has nothing in particular to do with diabetes. But we took insulin, and we looked at insulin directly by mass spectrometry. Now, what we're doing here is more or less a direct injection onto an electrospray mass spectrometer. To tell you the truth, what we do we actually put it on a small guard column, elute all the proteins together so we're not separating any proteins.

Okay; now, if we treat the insulin with a fairly high concentration of trichloroethylene oxide, we see all of these extra peaks. These all

correspond to modified insulin. And this is probably reasonably quantitative, because we're using electrospray ionization, so we're not really changing the sensitivity.

So what you can do here is integrate these, and you can tell how many of each adduct is being formed with the insulin, and in one shot, we can quantitate the binding to this particular protein. But we did another experiment, and we took this sample, treated it with mild base, 0.1 normal sodium hydroxide, which would not affect the lysine residues, but it would affect any ester groups; did this for 5 minutes; neutralized it, and you can see that most of these adducts, many of these adducts have disappeared.

So this would argue that many of the adducts are not to the lysine group, but they are to things like cysteine, tyrosine and serine and these would be unstable. Let's go back to this, now, and so, what about neutral pH? Well, we did the experiment again with insulin and also with

ACTH as another model, and even at neutral pH, you can see that the adducts are disappearing pretty rapidly; in fact, they have a half life of about an hour under these conditions.

And we get down to this level. This residual level presumably corresponds to the lysine derivatives, but we've lost these other adducts. So they're probably formed with the hydroxyls, that is, serine and tyrosine and perhaps cysteine. So let me go on and okay, what does this mean? Well, what we did here, we picked two enzymes, and these are known to have critical lysine groups. And we modified these with enough trichloroethylene oxide so we knocked the activity down by 50 percent.

We didn't want to completely obliterate it, because we wanted to see if this was reversible. So we knocked this down by 50 percent, and you can see with time, the activity of these enzymes stays down, again because we've probably hit a critical lysine group.

Now we went to some other enzymes. Chymotrypsin is a model enzyme. It has a reactive

serine group. D-Amino acid oxidase has an important cystine group and tyrosine group, and papain has an important cysteine group.

So then we do the same experiment, and there's a bunch of baggage here because we're also using some model asolating agents, but the activity comes down halfway with the trichloroethylene oxide, and now, it comes back over this time period; same thing happens here; same thing happens here.

So in fact we're regaining our activity again with a half life of about an hour, and this corresponds to what we saw when we were doing the mass spectrometry experiments on the stability of the adducts. So you can interpret this in a couple of different ways in terms of its relevance to toxicity, et cetera, but the point is we seem to have a lot of unstable adducts formed with trichloroethylene oxide, and if you go back to the old days when we started and the ways people looked at covalent binding of proteins, we would have missed these, because it would have taken us too

long to do the analysis with radioactive material.

We don't think that the epoxide is particularly genotoxic. We have tried Ames test, and there's a real response, but it's pretty low. We have done experiments with small oligonucleotides, again, using mass spectrometry, and one can find adducts. Apparently, they're with guanine; I won't go into why we believe that. But these are also unstable, and you lose these with a half life, again, of about an hour or actually less in this case. So we have unstable adducts formed with nucleic acids and DNA as well.

We have gone on, and I'll go through this briefly, but we've worked with perchloroethylene or tetrachloroethylene as well for comparison. Again, I think the literature had always postulated this epoxide as being critically on the way to the acid chloride and trichloroacetic acid. We don't think that's true; we think that that's also the case here as with the P450, where we have an intermediate that can form the epoxide and also this as well.

This is certainly capable of reacting with lysine as well, and we have verified that, and we have also worked out this pathway. We didn't do this in as great a detail as we did with the trichloroethylene oxide, and I'll come back to this later. Turns out that the critical intermediate here seems to be oxalyl chloride that's formed in the hydrolysis, and that's an interesting molecule.

This is something you can buy from Aldrich and work with, and we found some interesting things, for instance, a reaction with phosphate as well as with lysines, and this also undergoes this carbon-carbon bond scission to give carbon monoxide then.

So what we've tried to do is pull together a number of studies that we've done over the years with a number of different compounds. Here's vinyl chloride, which is sort of off by itself in terms of its decomposition products from the epoxide. But with these compounds, we can see that, if you will, this carbon here, which is bound to those two halogens, always seems to come out as carbon

monoxide and then, depending on the valence state due to the amount of chlorine substitution, we wind up with other products in a different oxidation state. Here, we get formaldehyde; here, we get formic acid, and here, we get carbon dioxide as well as these two carbon hydrolysis products as well.

So we think that we have a fairly good grip on understanding what some of these reactive metabolites that are produced, how they really behave in terms of their chemistry.

Now I mentioned before that we really characterized the reaction with perchloroethylene in terms of going through these acylhalides, and this seems to be reasonable. In fact, it turns out that if you do these reactions in vitro and phosphate buffer, that's really a problem, so we've been avoiding phosphate buffer, because you're forming something called oxalyl phosphate in this particular case. We've actually characterized, identified, isolated this and identified it and then looked at its reactions as well.

You can see the formation of oxalyl phosphate, and you may think that this is just a curiosity. It turns out, if you go back in the literature and look at what a lot of the people have done over the years, historically, a lot of people have done 100 millimolar phosphate reactions, and we know now, in fact, this explains some of the discrepancies we saw early in our trichloroethylene work, where some of the intermediates would disappear if you used phosphate buffer instead of something else.

So you can see the oxalyl phosphates forming. We've then taken the oxalyl phosphate. It is not reactive, so it won't react with lysine, for instance. So this seems to be an artifact that's in a lot of the literature, and that's something that we need to be aware of. What does all this mean? Well, we can--we've learned some things, and some of these have practicality; some are more basic and may not.

We know that cytochrome P450 2E1 in humans or in animals seems to be the main enzyme involved

in trichloroethylene metabolism; it yields mainly a chloral or chloral hydrate; it does yield some of the epoxide. But the epoxide is not the precursor of the chloral. The trichloroethylene oxide seems to be the major reactive metabolite of interest, at least in the oxidative system. This rearranges to an acylhalide, and that goes on to react with nucleophiles.

So we can understand what they are. In proteins, the reaction with a lysine will give you an amide, which is a reasonably stable entity, and you get either the formyl or the dichloroacetyl derivative. You also seem to get a lot of reaction with these alcohols, with cerine, threonine and tyrosine, as I pointed out.

We haven't been able to characterize these too much. We've known in this work that if you do the reactions, we can make these amino acid derivatives with the epoxide, but they won't survive overnight in buffer, so we can't do much with them. And as I mentioned, we also get reactions with phosphate buffer. We're not sure

what that means in terms of potential reactions with the phosphates on DNA.

This issue of the semistable protein adducts with these alcohols is one of interest. We've shown that this modification can affect biological activity transiently, and the question is does the existence of these raise or lower the risk?

You can look at these things two different ways. You can say, well, these things aren't around for very long, so this isn't really very important. On the other hand, you can say, hey, we're totally underestimating the amount of covalent binding, and even in this hour or so time frame, hour or two time frame, we could have something going on in terms of disrupting regulatory processes.

Finally, any PBPK models and other things should really not be based on the epoxide being obligatory in terms of going on to chloral or trichloroacetic acid or ethanol. The final point I'd like to make is that we need to consider the

human variation in the overall activation process if, indeed, this activation is important for the toxicity.

I have not dwelled on exactly what the manifestations of the covalent binding might be. You can envision a number of different things, not necessarily related to cancer but perhaps to other toxicities in terms of immunology, cardiotoxicity, things like that.

So with that, this is kind of dark, but, most, well this work has been going on for a long time. It was really started by my first graduate student, Randy Miller. Most of what I talked about today was done either by Hongliang Cai, who's at Pfizer now, and the latter stuff was done by Tadashi Yoshioka with the perchloroethylene, et cetera, and thanks to my benefactor, the NIH, and I'm just out of time I guess.

Thank you.

[Applause.]

QUESTION: Two questions. Dick Bull, MoBull Consulting.

Do you have an estimate of how much of that epoxide would go to dichloroacetic acid versus--

DR. GUENGERICH: Oh, yes.

QUESTION: Give it relative to the chloral going to trichloroacetic acid.

DR. GUENGERICH: Well, it's a major product. You caught me a little off guard here, but I think it's about 30 percent of the epoxide. Now, the epoxide is a minor product compared to chloral, so we're probably talking down, you know, 5 percent of what the chloral pathway would be.

QUESTION: The other question with this phosphate reaction, how much reaction would you expect with inorganic phosphate in the cell?

DR. GUENGERICH: Probably not that much. I don't know what the physiological concentrations of free phosphate are. They're not 100 millimolar. So it's--the point is not that that's going to be an issue in your body, but I think it's probably been a player in terms of a lot of the literature that is out there; in fact, I would say that

probably most experiments that used phosphate buffer probably are underestimating the amount of covalent binding in various studies.

QUESTION: David Jollow, Medical University of South Carolina.

I had the same question as Dick's first question but with an add a little bit. Can you imagine any situation where you may change the disposition of the oxide into the chloral hydrate versus the--sorry the P450 intermediate into the two pathways? Can you imagine any differences within the cell that may affect that breakdown ratio?

DR. GUENGERICH: Probably not, because we think it's happening inside of the P450. Now I will say this, as you go to development of P450s, that ratio probably varies, and, in fact, there's well this is non P450 enzyme, a bacterial enzyme called methane monoxygenase, which will also do this kind of chemistry, and that enzyme apparently makes all epoxide and no chloral.

So we think that different P450s will

vary. I doubt if the ratio would be sensitive to anything else that I can think of.

QUESTION: So you wouldn't expect an effect of polymorphisms within the 2E1s?

DR. GUENGERICH: Well, you know, it could happen. When I said different enzymes, when you have a polymorphism, if it's in the coding sequence, I suppose that could do that, because effectively, you have potentially a different enzyme.

QUESTION: John Lipscomb, USEPA. Two quick questions: as we begin to develop more and more sophisticated physiologically based pharmacokinetic models, one is tempted to consider the lipofilicity of a compound and determine the differential partitioning of that compound into the aqueous versus the lipid medium of the cell and the endoplasmic reticulum, also known as microsomes when isolated.

Do we know where the active site of the P450 2E1 is? Does it reside in the part of the protein, in the membrane, or in a cytosolic-exposed

region?

DR. GUENGERICH: The short answer is no. We have some hunches, though, from--well, we certainly don't know with P450 2E1. There are some indications from the few mammalian P450s that have been crystallized. In the case of P450 2C9, it looks like it may be used, the quote, entry channel may involve interaction with the membrane. But even that is a little fuzzy. So it's probably premature to try to deal with that too much right now.

QUESTION: My last question gets back to some ongoing conversations about the impact of genetic polymorphisms on susceptibility and risk, and I was wondering if you could clarify your comments; specifically, are you aware of any polymorphisms in CYP 2E1 that affect the functional component of the protein?

DR. GUENGERICH: The answer is no. There are a bunch of--a number of polymorphisms that have been identified in P450 2E1, and my colleague at Vanderbilt in clinical pharmacology Grant Wilkinson

has looked at some of these or at least some of the common ones. They've looked in vivo with real people looking at chlorazoxazone metabolism as an indicator, and they really haven't seen any differences in the pharmacokinetics yet.

Does that mean they don't exist? Well, we're not sure. Now, when I talked about variation in the population, this is not necessarily due to genetic polymorphism. There are a number of other factors; you know, it depends whether people are exposed to other inducers or, you know, how much they drink, things like that. So there are--just because something is not--a variation is not genetic, doesn't mean it's not real and important.

QUESTION: Thank you.

DR. PREUSS: Last question.

QUESTION: Stott, Dow.

Fred, I thought I heard you answer a question that the phosphate interaction in the early work would have underestimated--or overestimated--

DR. GUENGERICH: Well, a couple of things.

Remember in, you know, Randy Miller's thesis work, we knew that if you did the breakdown of the trichloroethylene oxide in phosphate buffers, some of the products disappeared. We understand that now because they were getting reaction with phosphate.

The point I'd like to make, though, is that if people did reactions, for instance, and measured covalent binding to proteins in the presence of high concentrations of phosphate, and typically a lot of these experiments used 0.1 molar phosphate, you would be basically squelching some of the reactive intermediates.

DR. GUENGERICH: Thanks.

DR. PREUSS: Thank you.

Our next presentation is by Jeff Fisher from the University of Georgia, who is going to update us on PBPK modeling.

DR. FISHER: Good morning. The talks yesterday--some of the new effects about trichloroethylene from a researcher perspective, I noted one effect that wasn't mentioned that has to

do with Dick Bull. When he started working, his hair was black. Now, all of a sudden, it's gray.

[Laughter.]

DR. FISHER: There are some confounding factors. Age, I guess, could be one. But I've noticed that trichloroethylene has caused tension and confusion also.

[Laughter.]

DR. FISHER: And it still continues.

I'm going to talk about some work that's been done recently at Georgia. It's been supported by DOE through a subcontract with the Medical University of South Carolina.

I was asked to comment on what's been going on since the monograph on trichloroethylene in 2000. I'm going to do that. I'm going to talk about some work that was done with trichloroethylene itself in the rat based on data collected by Jim Bruckner's laboratory and then some information that we collected on the ability of TCA to bind to protein in the serum from different species.

We have work ongoing now with DCA in the model development of DCA PBPK model in mice and rats, and it accounts for zeta glutathione inhibition. Then, I'm going to end up and talk for a few minutes about what's really going on with the models, and one of the big issues that's come out since the monograph is the harmonizing of the two models, Harvey Cole's and my model, and that we use different data sets. And I'm going to talk just a few minutes about what's going on with that. We think that's an important contribution to the EPA's effort.

Dr. Deborah Keys is working at the University of Georgia, and she published a paper recently on trichloroethylene and a lot of the solvent models we usually use, four compartments, five maybe. Well, we had a large data set, and we used several compartments. And Deborah is a biomathematician, so she spent quite a bit of time understanding, well, does it matter if it's a four compartment model or a nine compartment model?

And to answer that question, it really

doesn't matter for richly profused tissues. But along the way, we found out a couple of interesting things about trichloroethylene. We kind of knew that in the fat, you see we have an arrow going both ways.

We found that trichloroethylene loads into the fat rather rapidly but clears very slowly. We never really looked at the fat in a lot of the studies that we did before. It does have some implications for residual TCE for low level exposures and also about metabolism in the liver, which we used a two-compartment model in an attempt to describe the trichloroethylene kinetics in the liver itself.

First, the liver: to point out, usually it's assumed to be simple one-compartment, uniformly mixed liver, and we never collected liver TCE time course data. Jim Bruckner did. And so, we looked at the data, and we couldn't describe it with the models that we had published.

So Deborah Keys developed the next-simplest thing: a two-compartment model, in which

we assume that there is a deep compartment and a shallow compartment and that the metabolism occurs in the shallow compartment; that the deep compartment may contain liquophilic stores that exchange slowly with the blood.

Well, we did a better job. The blue lines represent the two-compartment simulations, and the red lines represent our old simulations.

Looking at the fat, we never collected fat either, and the fat clears very slowly. The blue line shows the new data sets with the model predictions assuming diffusion limitation, and the dotted line in red shows our flow-limited assumption with rapid clearance.

The implications for model development for use of PBPK modeling in risk assessment: the liver compartment is really insensitive to metabolism the way we've developed the model now, because we assume that the metabolism occurs in, quote, the shallow liver. The diffusion limitation in fat, however, is a sensitive model parameter. And that's a little more serious, I think, in terms of

making sure we incorporate that in the harmonization of the PBPK model for trichloroethylene and metabolites.

As I mentioned before, the idea of having multiple compartments or three or four compartments at least for richly profused tissues doesn't have much impact. And Deborah Keys did a statistical approach, cross-validation, to look at the effects of removal of a compartment, one at a time, something I would never do, probably.

Another ongoing project is dealing with trichloroacetic acid. The models that I have listed here were published and were used in the monograph. And we've modified them for TCA as a subcompartment or a submodel of trichloroethylene metabolism to include new information on binding, serum protein binding.

And this is taken from Michael Lumpkin's dissertation research. He published this paper recently. And he did equilibrium dialysis binding studies with sera from mouse, rat and human, and it's really interesting: there had been some

published work; Dick Bull had done some work and colleagues; we did some when I was at Wright Pat. But Michael did a really thorough job looking at a wide range of concentrations, which is on the X axis and the percent balance on the Y axis, and as you can see, in the mouse, the fraction bound is much less than in the human.

That's an important consideration for PBPK modeling because of trying to understand tissue dosimetry of TCA, an extrapolation of animal findings to human if you were to do that. For some people that might be interested in this, we don't have on and off rates. We solved the equation of equilibrium, and we used a formulation that was created at Wright Pat by John Frazier and Brent Foy.

So this is what we used in the model. It's as determined in vitro in the cells, equilibrium dialysis cells. To give you a flavor of the issue, all TCA and serum or whole blood is measured for most--I can't think of any times it's not--but it's total TCA but a lot of TCA is bound,

and some is free. So what we have here is one of our human exposures in a simulation of total and free TCA in blood, and total TCA is what we measured.

You see that in the red boxes and blue box represents measured free trichloroethanol, and this particular subject was exposed for four hours to trichloroethylene. This is the 1998 paper on a human PBPK model. Well, the point I wanted to make is the calculated free is much lower, and the implications for dosimetry are that the tissue dose may be level if only the free TCA is available for diffusion into the tissue such as the liver. So we are currently looking at the implications of that.

DCA: there's been a lot of data collected since 2000, quite a few studies at the Tel Northwest, where Dick Bull was. Irv Schultz and his colleagues working with rats, mice and most recently humans, and there's some human DCA kinetic data in the literature also.

What I'm going to present is a little bit of information on DCA. This model is not connected

to trichloroethylene. It's a DCA standalone model, if you will. And DCA has been published as a PBPK model as a subcompartment of trichloroethylene, and in those models, the inhibition of its metabolism is not accounted for, and there's been quite a bit of work done on the metabolism of DCA, as you probably know. Several of you--Anders Lab, Stackpool in Florida.

And I'd like to point out that what we have here describes inhibitable metabolism, zeta glutathione metabolism, but we're also proposing, there's also another pathway that's a minor pathway, but it doesn't appear to be inhibitable--not inhabitable.

[Laughter.]

DR. FISHER: So this is from a modeling kinetic analysis; you know, we have not looked at the pathway and tried to identify metabolites but a kinetic analysis.

And the zeta glutathione passway is recognized as the major pathway, and that's how we're describing it in the model. It's just under

inhibited condition, there's still clearance of DCA, and we're proposing that there must be another minor pathway. The minor pathway becomes much more important under severe inhibition of the zeta pathway.

In this model, we have included data collected by Anders Lab colleagues on actually monitoring the activity as well as the protein content of zeta, and this is at one dose, so we have developed a function described as a Vmax that changes with time. It's inhibited with time and to reproduce this time course for zeta glutathione.

To give you an idea how pronounced the inhibition can be, here's a simulation of a data set collected from Batel Northwest and published recently. In a naive rat, a 20 mg per kg IV dose, here's clearance kinetics, here's a model or model prediction. As it stands now, if you pretreat the animals, here's pretreatment with 200 milligrams per liter for 7 days and then do the IV dose, see, the clearance is inhibited.

This has been known for quite awhile. It

was observed by Dr. Stackpool in human studies with terminally ill children. So it is a significant inhibition.

I'll show you just one more data set. This is over inhibition and then dosing with a wide range of doses. We do reasonably well at some of the doses with our model describing the clearance kinetics of DCA.

And drinking water study, one more example where mice were put on a high dose, 2 grams per liter, one of the cancer bioassay levels for 14 days and then removed and put on clean water, and we monitored clearance kinetics of DCA. This is unpublished data from Wright Patterson.

Well, what about trichloroethylene going to DCA? The epoxide could be, you know, a good pathway to describe mathematically for formation of DCA. We have a real problem, still, trying to provide estimates of the formation of TCA from animals that are dosed with trichloroethylene, and the chemist, Dr. Michael Bartlett, and a graduate student, Amy Dixon, have been working for two years

with DCA, and it has included co llaborations with CDC chemists and EPA chemists in Athens, Georgia.

And in water, they do real well. And there's a paper in press using LCMSMS method. However, we're still not sure about animal studies. When we dose animals with trichloroethylene and then try to determine how much DCA is there, I thought I would have a better story to tell today, but we think there's still artificial production of DCA in our animal studies. One thing they're doing is spiking samples with DCA after they pull the sample, and they can artificially produce DCA by adding TCA.

So I don't have anything promising to say right now about studies trying to estimate the amount of DCA that's formed in the whole animal. As some of you are probably familiar with in the literature, we found artificial production of DCA in the presence of high levels of TCA, which is what occurs when you dose animals with trichloroethylene.

The most recent data sets, 1997 and up, we

added lead acetate to the samples to quench what we hoped would quench the conversion. We think the data are better, but I'm not sure if it's 100 percent, if we are still not dealing with some type of artificial conversion of DCA. And as you heard yesterday, DCA is a pretty important issue for trichloroethylene, even if it is a minor metabolite.

And I think, you know, we need to figure out how to deal with uncertainties associated with DCA, and we can do things from a modeling perspective and probably put boundary conditions on it, on the information along this pathway.

A couple minutes about the harmonization of the models: in the EPA risk assessment for trichloroethylene, there's three or four chapters on the models, and Frederick Blaws' use of Bayesian analysis with the models, and Harvey and I used different data sets, so there was some overlap. But we had different data sets, and so, the models diverged in outcome, especially if you took it to the point of risk assessment. And, of course, that

would cause a lot of concern.

And we knew it afterwards, but we didn't work together on this effort. Harvey had done it a few years ago as a contract effort for the USEPA, and we were using new data that we were collecting, both human and animal data, and I didn't go back and use all the old data sets.

So now as of, like, a month ago we met in Cincinnati we met with USEPA people and involvement of USEPA and Harvey and I to try to come up with a model structure for trichloroethylene and metabolites that would then be used in some capacity by USEPA scientists in their next document on the health risks posed by trichloroethylene.

There are lots of questions that come up, and we're dealing with some of the technical issues now. I would say the binding, serum protein binding of TCA is probably important for species extrapolation of information. DCA is an uncertainty quantitatively, for sure, and trying to deal with how much goes down that pathway because of the analytical methods and the shortcomings.

And that stuff is in the literature, and I think most of the people who are doing work with DCA know it and especially trichloroethylene literature.

That's what I wanted to say today. Thank you.

[Applause.]

DR. BULL: Dick Bull, MoBull Consulting.

One of the things, and I don't know if you've noticed, in Irv Schultz's publications, because I'm not sure which paper he mentioned it in, but that GST inhibitable metabolism disappears with age. So you get evidently an isoform switch, or you depend on the noninhibitable pathway, whatever it happens to be as you get to a rat that's--I can't remember if it's a rat or a mouse, but after you get past a year of age or something.

DR. FISHER: Yes, I think it's in the rat.

QUESTION: In the rat.

DR. FISHER: We haven't dealt with that.

QUESTION: So it's not a constant issue throughout, so somehow, you're going to have to take that into account.

I had another question but I don't remember what it was. Oh, I know what it is.

DR. FISHER: It's the effect of trichloroethylene.

[Laughter.]

QUESTION: Right, along with the gray hair.

How much of the difference between the two models is actually the data or things that were incorporated in the two models as a result of starting with the two different data sets? Is it a structural problem with the models, or is it that came out of the--

DR. FISHER: Well we used different structures; that is, in some cases, some of the compartments were different. But Harvey also included lung metabolism, which I didn't include, and included glutathione pathway with an interest in kidney, which I didn't include. I was focused on liver pretty much. So it's combining the best of both with new information.

QUESTION: Hugh Barton, USEPA.

Nice talk. One suggestion for thinking about the liver is to perhaps think about the regional distribution of the 2E1. One thought that popped into my mind as you were talking was since the blood comes in periportal, and it's, I believe, dominantly centrolobular, whether essentially the periportal region serves as your deep compartment; in other words, an area where it would be stored but not metabolized, and then, the centrolobular region would serve as the shallow compartment, as you have modeled it.

I'm not sure whether that would work if you tried to model that geometry. I know for benzene and its metabolites, some of the work that was done in CIIT showed that modeling the regional distribution of the different enzyme systems was actually quite important. So I would just toss that out as a suggestion.

I also think the blood binding protein work, as you indicated, will be quite important to incorporate in the model.

DR. FISHER: Thank you; we know that there

are more sophisticated approaches at handling the liver, and I think it's useful to go there. I just wasn't planning to.

[Laughter.]

DR. FISHER: If I keep working on trichloroethylene, I probably will be there.

[Laughter.]

QUESTION: Lipscomb, EPA. Yes, and your hair is going to get gray, too.

[Laughter.]

QUESTION: Thank you, Dr. Fisher.

I had a question about species differences relative to trichloroacetic acid binding. If we assume that the liver is the site of production for trichloroacetic acid, one would assume, then, that TCA diffuses from the liver into the blood, and that's where we see the species differences in binding.

My first question is the straightforward one: do we know which protein is binding TCA in the blood?

DR. FISHER: No, there was a correlation;

we measured the albumin content in the bloods, and Michael found a higher correlation with albumin versus total protein. But no, we never looked at that.

QUESTION: A suggestion which is obvious to you would be the application of techniques like two-dimensional gel electrophoresis to identify the protein that we used to do with Frank Woodsman and others.

Here's the other one that's not quite so straightforward. If the liver is the site of production for albumin, how might that affect your consideration of TCA mobility from the blood to the liver and subsequent sequestration, so to speak?

DR. FISHER: Say that again? If the --

QUESTION: If the liver is the site of production of albumin, and if albumin is one of the proteins that's important in binding, how might you consider that?

DR. FISHER: I have thought about that. I mean, it could be binding in the blood supply, in the blood of the liver, or as it's leaving the

liver, and that's a refinement that would be important if we were looking at rates, rate on, rate off.

You know, our approach was at equilibrium, under conditions of equilibrium, but a more refined look would want to address the production of albumin in the liver and where TCA is being produced also and the interaction of the protein and TCA in the liver.

QUESTION: Thanks.

QUESTION: Louie Blumen, Dow Chemical.

Yesterday, there were results presented which indicated a different response between male and female rodents, and in epidemiology, there are also, although inconsistent, suggestions for difference in response. What factors in your PBPK model would be able to explain this?

DR. FISHER: We're not going to offer you much help there with human data and kinetics, controlled human exposures to trichloroethylene. The differences between adult males and females, there may be modest differences with

trichloroethanol production. But we didn't see big differences between males and females from a kinetic perspective in looking at TCA and trichloroethanol, free trichloroethanol in blood and urinary secretion of the glucuronide of TCOH and TCA.

It wasn't as great as I would have thought myself when we were studying, doing the human study, based on what was in the literature with rodents. But there's high variability in the human data also, I should note, just looking at time courses of the metabolites in humans exposed to the same concentration of trichloroethylene.

DR. BULL: Dick Bull again, MoBull Consulting.

One of the things that I think is different, an important difference between humans and the rodents is the half life of trichloroacetic acid, and I don't think from what little I know about protein binding that that difference in protein binding is going to change the half life of trichloroacetic acid, so I've always had this

notion, never addressed it experimentally, that there's got to be some tubular reabsorption of trichloroacetic acid. Is anybody looking at that issue at all in human--maybe in all of the animals, but for a real polar compound, it's got a fairly long half life even in the rodents.

DR. FISHER: It does.

QUESTION: And I'm curious as to why, if the tubular reabsorption differences might be very important.

DR. FISHER: At the University of Georgia, Kathy White, the pharmacokineticist, is very interested in that issue alone about filtration and clearance of TCA and why the big difference between rodents and humans, and, you know, the anti-ion transporters could play a role.

At Wright Pat, John Frazier on a postdoc did profused liver studies with TCA, and they're proposing active uptake of TCA from a mathematical analysis of the data. But that has not been looked at closely yet. And the half lives are just remarkably different.

DR. PREUSS: Thank you.

Our final speaker this morning is Dr. James Bruckner of the University of Georgia who will talk about pre-systemic elimination of oral TCE.

DR. BRUCKNER: Okay; thanks. I think I'm sort of the second half of the tag team. Actually, I'm really glad to have Jeff come to Georgia, because I've been generating data now when my beard was the same color as my hair. Luckily, my hair hasn't changed that much, but my beard has turned gray.

But with Jeff coming, I guess I'm sort of the data generator half of the equation. I think we've actually, you know, we're beginning to see different things once you have really complete data sets. A lot of people, I know, have done modeling with other people's data or with, just, you know, really sparse sets.

One thing I wanted to do today, or the thing I wanted to do today is really is to take off in a different direction, and I just wanted to go

back over some of the work we've done on presystemic elimination over the years and end with what we're working on right now, and I'm going to take it from the standpoint of presystemic elimination or first pass as being a protective mechanism.

I guess the bottom line is we have trace levels of TCE and these other chemicals in our drinking water. Upon ingestion, you know, the question is going to be do those trace levels actually make it through the liver and the lungs and reach extrahepatic tissues? Doesn't help with inhalation, so we're just focused on ingestion.

Let's see, we can find--just for a quick review, of course, most chemicals that are absorbed from either the stomach or the intestines, which are just drawn partially here, of course, are absorbed into the mesenteric blood vessels which converge into the portal vein, and, of course, the portal vein then funnels these chemicals which percolate slowly through the lobules. So obviously, you have a very good chance of whatever

is absorbed from the majority of the GI tract to be actually taken up by the liver and then partially metabolized.

Of course, blood, then, from the liver then goes into the vena cava and then out to the arterial circulation. I guess there is a possibility of a first pass elimination if we're talking about trace levels just looking in the wall of the GI tract itself. Personally, not based upon data, but personally, I wonder how important that really might be if we're talking about TCE, which, like other VOCs, passes through membranes almost as if they're not there.

For example, we've dosed animals with trichloroethylene and these other VOCs, and we typically give them either in water or as an aqueous emulsion, since oils, of course, really inhibit their absorption or delay the absorption. And we see, after dosing a fasted animal with an aqueous solution of trichloroethylene, we see peak blood levels, arterial blood levels within --as short as perhaps 3 to 4 minutes or even as short as

2 minutes. So they're absorbed really quickly.

The second organ of elimination, of course, for volatile chemicals are the lungs. So, I'll talk a little bit today and some experience where we actually tried to distinguish how much contribution there is from the lungs and how much there is from the liver in total absorption or elimination of these compounds.

This work that we began back about 10 to 12 years ago, and I was on a couple of the NRC's safe drinking water committees, and at that time, for a lot of the volatile chemicals, the majority of the information as far as toxicology information we had was from inhalation studies, since those, of course, were of primary concern because of occupational exposures; in other words, most occupational exposures were inhalation, and so, the majority of the data that I had to work with on some of these committees was inhalation data.

And the big question we had then is can you extrapolate either from a qualitative standpoint or a quantitative standpoint to

ingestion? So, actually, what we have here is, I guess, the question that I began to work on back in the early 1990s as far as what can we do in terms of route-to-route extrapolation of inhalation data to ingestion.

Okay; the approach that we took, and I'll go through this with you, is to administer equivalent doses by inhalation and by gastric infusion over the same time frame. So our first problem, then, was to actually determine with inhalation exposures how much of the dose was actually absorbed systemically.

And this is the diagram of the system that we used at that time. We took male Sprague-Dawley rats, and over a period of days, we tried to train these animals to stay within a restraining tube and also to breathe through a little mask that was molded and fitted to their face. To that mask--this is really drawn out of proportion, but we had a really tiny one-way breathing valve. So the chemical actually would come in here, okay? A little flap would open when the animal inspired,

and then, when he expired the flap would close; this flap would open, and then, the chemical would go out this way.

And on this little, tiny valve, which we kept as small as possible to minimize dead space, we could then take, from these sampling ports, automatically we had set up that we would actually take--we would measure the difference between the inhaled concentration and the exhaled concentration, and the difference being the amount that was taken up by the animal.

A couple of other things: when we were doing our training, we tried after, well, after, you know, a period of months, we got more successful. We measured the respiratory rate, the minute volume and the blood pressure, and we, after six or eight months of trying this, we actually brought in some animal behaviorists, and we were actually able to get, after a period of training, we got fairly stable blood pressure, respiratory rate, minute volume that weren't that different. They were a little higher, but they weren't that

different from what we thought it would be in an unstressed, you know, unrestrained animal.

The other thing we did was before these animals actually went into the tubes, usually 24 to 48 hours before that, surgically, we put in a carotid artery cannula so we could actually take, at the same time we were taking the breath samples we could take serial micro blood samples and measure the content of the VOC by headspace analysis.

This is just a simple calculation, really pretty straightforward. We had the inhaled concentration; then, instead of the exhale concentration, just took into account the dead space in the upper respiratory tract and in the valve itself in terms of a minute volume, and by this way, we were actually able to plot the cumulative uptake or total uptake of these chemicals.

This is an experiment in which we--actually, this time, you have to look at it a little bit differently. Okay; TCE, in my vernacular stands for trichloroethylene. What we did, here is an experiment. I wanted to compare a

well metabolized VOC with a poorly--TRI here is 1-1-1 trichloroethane, which is, of course, a poorly-metabolized chemical. So, here, we plot, then, we had two hour inhalation exposures in this experiment, and we took--here, it's shown at 10-minute; we actually did it at 1 minute intervals, and so, we had plots of cumulative uptake of trichloroethane.

As you'd expect, for a well-metabolized chemical, because metabolism acts as a bit of a sink, you have greater uptake of the trichloroethylene. And you can see, after two hours of inhalation, you have a total uptake of about 2 milligrams of trichloroethane and something less than 3 milligrams of trichloroethylene. This turned out with the rats that we used, which were fairly large, to be a dose of about 8 milligrams per kilogram of body weight.

And here's the type of data, then, we were able to obtain from these studies. These, now, in rats that are not restrained, but we did monitor blood pressure and those things, and they're a

little bit lower than the actual animal in the tube.

Okay; so we have again, we have taken serial blood samples, so we have, with the inhalation exposure, very rapidly uptake of the chemical in the blood--this is carotid artery; okay, the blood concentration increases very rapidly, and then begins to reach a near equilibrium or steady state, doesn't quite reach but begins to approach that.

Okay; gave this 8-milligram dose, which was, you know, what we determined with the inhalation exposure, and you can see what a profound difference. This is the arterial blood concentrations in the animals. These were administered, I should say was with a gastric canula, so we had--it was surgically implanted, so we had the chemicals basically going in at the same doses, hopefully, at about the same place.

The problem, of course, is that you have a chemical being absorbed at a different rate, at a different portal and entering the systemic circulation at a different point. But this is the

best way that we had to come up with comparing. So you can see, there's quite a difference between C -maxes and between area under the curves for the two routes of administration.

We did a number of these chemicals--I just wanted to show you another couple. This was with carbon tetrachloride inhalation exposure for 2 hours of 100 parts per million, and with the same type of setup, we determined the total dose was 17.5 milligrams per kilogram. This, of course, is the inhalation arterial blood levels, and these are the blood levels in the animal getting it by gastric infusion.

A little bit different pattern; this time, of course, carbon tetrachloride is not as extensively metabolized. With this dose, we don't think we were getting a lot in terms of toxicity, because we monitored that in the animals. Of course, carbon tet, you know, kills 2E1 and most other P450s, but at this dose, I don't think we were hopefully getting too much.

And anyhow, you see with the longer half

life and the slower metabolism, it actually reaches the blood levels in the inhalation animals, and they decline at about the same rate. But still, you have quite a difference here between area under the curve for the gastric animals and for the inhalation animals.

This work, just to give credit, was actually supported by a couple of cooperative agreements between what, at that time, was EPA's HERL in RTP and our group. It did give us, I think, a pretty good handle on just how much of an effect that first pass elimination can actually have on arterial blood levels.

Okay; this compound, this was work we are actually just now completing. I wanted to actually, since we had such a big difference in how much reaches the arterial circulation, I wanted to look and see how much difference that would make for an extrahepatic organ in terms of just cytotoxicity. Since trichloroethylene doesn't really do very much, and it's not very exciting as far as cytotoxicity, I went and picked 1-1

dichloroethylene; okay, 1-1 dichloroethylene, of course, goes through probably an epoxide which is detoxified and conjugated with glutathione.

It's quite toxic to a number of organs in the body including not only the liver but the lungs and the kidney. So we picked in this particular study just to look at kidney toxicity.

Results are pretty predictable. This is time, instead of a linear scale, we have a log scale, but you can say the typical response, with inhalation exposure, this was 300 parts per million, again for 2 hour exposure. And the dose, we determined, during that two hours, was 30 milligrams per kilogram. So again, we gave that dose over the same time frame. And you can see, you know, quite a difference again, between the area under the curves and the peak blood levels.

We have quite a bit of data. I just wanted to pick out one slide just to show you as an example of the difference in toxicity. So, we're going to compare the toxicity, then, in these two groups.

Okay; here, we have just one particular parameter. We looked at quite a number of them. This is gamma-glutamyl transpeptidase, which, of course, is just a brush border; it is the enzyme which is released into the urine; okay, if you take a look, what we did was measure the release during the first 12 hours after dosing and then the second 12 hours, so the two together would be a total of 24 hours.

If you look at the gastric infusion route, you can see that levels are not very exciting. But when you look at what happens with that same dose given by inhalation exposure, you get just a tremendous wipeout of the kidney. Pathology showed just, you know, total destruction, as did all of our other indices.

One other group we had here, PO stands for oral bolus. And one thing we were thinking at this time is if you give this entire dose at one time orally, then, the amount of the chemical coming into the liver is going to exceed the metabolic capacity, and a lot of that will pass on and cause

toxicity. I mean, you can see in this particular experiment that really not that much happens when you give it as an oral bolus.

Okay; the next but--not the next but another series of experiments that we did where we actually wanted to look at the actual contribution of the liver and the lungs to first pass elimination of a series of chemicals. What I'll show you now is just some of the data that we have for trichloroethylene.

Okay; this is our experimental setup, so, this has been predicted by PBPK modeling, but I think ours was the first work we actually did direct measurements to try to get a handle on this, okay? These, again, in all cases, we've used--we stayed with male Sprague-Dawley rats. So what we did in different experiments, these are with cannulated animals; in the first instance, we administered the trichloroethylene; this is now as an aqueous emulsion, okay?

We administered this through the jugular vein. In that same animal, we took blood samples,

then, from the carotid artery. So, of course, what we're measuring, the drop in blood concentration between this point and this point would be due to pulmonary elimination of trichloroethylene.

The next experiment or the next step would be we actually administered the trichloroethylene through the portal vein, and then we measured in the carotid artery, so this would, if you measure here and look at the drop in concentration here, you're getting total first pass elimination, so you simply subtract the lung from the liver, and you get hepatic first pass elimination.

A couple of other things we did: we administered trichloroethylene as an oral bolus and measured in the carotid artery. And then, to get intraarterial, we administered trichloroethylene in the carotid artery, and this sampling site was in femoral artery. So that was our intraarterial. So what you would do is to basically measure the area under the curves for each of these.

Here's what the plots look like. Okay; here is, of course, the blood concentration on a

log scale versus time after injection. Open circles represent what happens when they administer it into the carotid artery and sample from the femoral artery. This, of course, is trichloroethylene itself.

And the closed circles are what happens when you administer into the jugular vein, so this difference is, of course, due to pulmonary first pass elimination. And then when you administer into the portal vein, the difference between this curve and this curve would be total presystemic elimination.

The dose you see here, we tried to go down to as low a dose as we could, and this particular experiment was done with a dose of 0.71 milligrams per kilogram of body weight.

Okay; this is the table which--we gave a series of doses; the lowest dose we worked with here was 0.17 milligrams per kilogram, so you can see the dose has increased up to--the maximum dose in this experiment was 16 milligrams per kilogram. At these lowest doses, we weren't able to get the

whole time profile; in other words, the doses were so low, and our analytical sensitivity was such at that time that we actually couldn't get a complete area under the curve or time profile.

But when you look at these other doses, you can see that this is, of course, represented as a percent of the administered dose. You can see that the amount that's eliminated is relatively constant, and I think back to a paper of Mel Andersen's which I think he's actually felt that the primary determinant of pulmonary elimination was simply the blood-air partition coefficient, and he proposed that the pulmonary elimination, first pass elimination, would be independent of the blood concentration. So these data sort of support that position.

On the other hand, when you look at hepatic first pass uptake, it's highest, as you expect, at the lowest dose. And as you increase the doses, it actually, of course, diminishes as you saturate metabolism, so the liver becomes, of course, less efficient in eliminating the higher

doses. Now, remember, these are oral bolus doses.

And then, finally, at the bottom, total presystemic elimination. Now, what we wanted to try or I wanted to try to do was to get as close as possible to real life situation. And I guess one other thing to keep in mind is that most experiments, although we're now giving trichloroethylene in drinking water or even microencapsulated, but the majority of our database, particularly in the cancer studies, of course, involved oral bolus administration. So there's a question here of how realistic oral bolus doses are compared to environmental exposures in drinking water.

So the thing we wanted to look, which we're just now beginning to look at, is to look at the influence of the rate of administration. Of course, the rate at which the chemical arrives in the liver is going to be a major player in how efficient the liver is in taking the chemical up. This is the statement that Mel made back in 1981, and then, talking with them just a couple of months

ago, I think he said he didn't know, still, of any data that was available, you know, hard data available to support that.

But obviously, this becomes very important, I think, when you start thinking about risks to organs other than the liver, either cancer effects or noncancer effects. So, I guess, with trace levels, I guess the question is does any of the trichloroethylene actually make it through the liver and reach something like the lungs or the kidney or the testes, where you have 2E1 ready, you know, it's important to metabolic activation?

Because our objective here in this experiment, and these were just going on, and I'll show you just the preliminary results that we have, okay, the key word here, of course, is rate of administration. So what we did was to use the two extremes, an oral bolus versus a gastric infusion.

Again, we used trichloroethane as a poorly metabolized halocarbon and TCE as a well metabolized halocarbon. Experiment, again, our favorite animal; okay, the trichloroethane, we gave

two doses, 8, and 48 milligrams per kilogram. The key is an oral bolus and simply over two hours by gastric infusion. Same thing with trichloroethylene; the doses were just a little bit different than for trichloroethane.

Methods: we took serial blood samples again from these animals, and by derivatizing trichloroethanol and trichloroacetic acid, we could measure these as well as the parent compound in the same blood sample of about 10 microliters. So we could take a lot of blood samples from the same animal over an extended period of time. Kathy White, the pharmacokineticist, used Win Nonlin in her pharmacokinetic analysis.

Okay; this is trichloroethane in the two doses that we used: oral bolus and gastric infusion, and the interesting thing here is that trichloroethane seems not to be eliminated or taken up by the liver to any extent at all. There is some uptake in this group and I expect from this one; again, this is preliminary information, that because of pulmonary first pass, I would expect

that perhaps 10 percent of the dose might actually be eliminated by the lungs, very little by the liver at these doses.

Okay; trichloroethylene, okay, 10 in the 50 milligrams per kilogram dose given as a bolus and given by gastric infusion; of course, bioavailability again is intraarterial versus these oral administration routes.

These data, I have a hard time believing right now. With this high a dose, I'm surprised in these results. And I think one of our problems may have been looking back at the data, is that I think the analytical people here, the technicians, actually, I think were confusing maybe chloral hydrate with trichloroethylene. There was some overlap as I look back at these chromatograms.

So this is a work in progress. One thing Jeff didn't mention is that we had an explosion and a fire in my laboratory back at the end of September, and so, these experiments were done just prior to that time. The lab is going to be ready for us to move back in, I'm told, the 1st of April,

so we haven't been able to go back and redo these experiments.

Okay; we've also looked at the metabolites in these animals, so this is just the low dose, now, of trichloroethylene, the 10 milligram per kilogram dose. So we just had the time profile with the oral bolus and then that same dose given over a two-hour period by gastric infusion. And since trichloroethanol has a fairly, relatively short half life, you can see that the area of the curve is going to be substantially less than it would be with the oral bolus.

So I guess here, we were saying, okay, so, the amount coming into the liver, we're wondering how much difference that's actually going to make in terms of metabolite levels. And you can see it does have a pretty pronounced difference here. Always, the area under the curves are very different.

Let's go to the trichloroacetic acid. Okay; this is a little bit more confusing. I should have just put the one dose on this one, but

let me back up just one second. Okay; look at the pattern here. This is, of course, the low dose, but with the metabolite with a fairly short half life you can see that it's eliminated very quickly, and when it's coming in slowly in the liver, it has a fairly short half life. You can see that it never comes up near the oral bolus levels.

So if you contrast that with trichloroacetic acid with its long half life, this is the 50 milligrams per kilogram bolus dose. The orange is the gastric infusion dose. So there is obviously less of a difference in terms of total area under the curve. The green represents the low oral bolus dose; this represents the low gastric infusion dose. So, less of a difference on trichloroacetic acid.

One thing that I was kind of surprised as I really got into the literature and tried to understand trichloroacetic acid when we were doing the binding studies is that I discovered that you have a really efficient carboxylate transporter that can take one and two carbon compounds like

trichloroacetic acid, and very quickly, bidirectionally, you can transport it in and out of most tissues including the liver, which means, of course, that the trichloroacetic acid that's formed in the liver and transported into the bloodstream is available for transport back into the liver again, so it's not gone, you know, once it leaves the liver.

I always felt that since it was ionized at, you know, at physiological pH that that wasn't the case, but on looking into it, I think that it's freely diffusible--not diffusible but transportable from the blood back into the liver and probably lots of other tissues as well.

The area under the curves there are very different, depending on the rate of administration.

So sort of in summary, this is just kind of where I'm headed, I think. I guess the question is when you really get down, now, with our limit of sensitivity, we're able to--I think we're now down--I'm not really--I need to be sure of this, but I think we're down in the neighborhood of 10,

probably 10 nanograms per ml or 10 parts per billion when we're actually taking blood or tissue samples from mammals that are dosed.

I think we can probably go down another three or four fold in our analytical techniques as we go to the GCMS technique. So I guess the question is, if trichloroethylene, when taken orally, doesn't reach extrahepatic organs, I'm wondering, you know, of course, how that's going to affect both the noncancer and the cancer risk.

So this is our focus now. We need to go back and repeat these last experiments but actually look at some different dosage regimens, which, you know, I guess the question is, you know, what is the typical dosage regimen for somebody drinking water? I drink Dr. Pepper also as an IV infusion during the day. I really do.

[Laughter.]

DR. BRUCKNER: And so, the question is what is characteristic or typical of human exposure. Here, I have the two extremes, I think: a two-hour gastric infusion and an oral bolus.

These are the people I would like to thank. Most important person in my life is this gentleman here. He's been with me now for 24 years. I still call him SM, because I have trouble with his name, as does everyone else in the laboratory. Michael Bartlett is the analytical chemist who helps Jeff and I. Cham Dallas did the inhalation experiments that I've shown you. Cathy White is the pharmacokineticist, and, of course, I'm surely glad that Jeff is here now so I can do something with all of these data.

We're now being supported, of course, by subcontract with Charleston, and, of course, Larry Moore and David Jollow and some of the other people from Charleston are here, so we're all a happy family now. I don't have a picture like you did, but I wish I did.

Thanks.

[Applause.]

QUESTION: I presume or maybe should ask it as a question but Dick Bull MoBull Consulting, I remembered finally.

The trichloroethanol, how do you explain that one? And I'll give you what I think the answer is.

[Laughter.]

DR. BRUCKNER: Okay.

QUESTION: It's probably glucuronide, and it's going out the bile, wouldn't you think?

DR. BRUCKNER: Right.

QUESTION: Hi, this is John DiSesso from Mitre Tech systems.

One of the things that really differs between rats and humans is the function of the lymphatics in absorption from the intestinal tract. And certainly, things that are fat soluble tend to get picked up, at least in humans, in the bile, you know, it gets picked up in the lacteals, and it goes back by way of the thoracic duct. So it basically obviates or circumvents the entire first pass effect.

The difficulty is I don't know what happens in rats, and I'm not sure if you can even pick it up, because they're so small, and I don't

know what your vehicle was, and it can be confusing. So you may or may not pick it up in rats, but it may actually be a very important thing in humans that wouldn't be able to be modeled with the animal data.

Have you thought about how you might be able to deal with that, or has that ever even come up?

DR. BRUCKNER: Yes, sir, we've actually done some experiments. We haven't looked at trichloroethylene, but we've done carbon tetrachloride, some fairly extensive experiments, which is, you know, more lipid-soluble than trichloroethylene. And we found just looking at the effect of different dosage vehicles on lymphatic absorption, we found that with the aqueous vehicle, we had, of course depending upon the dose, we had less than a tenth of a percent of the total administered dose actually absorbed in the lymphatics.

When we gave the carbon tetrachloride in corn oil, we found as much as 2 to 3 percent of the

total dose was carried along with the corn oil into the lymphatics.

QUESTION: Then how do you think that will affect, you know, because humans have a much more fatty diet than rodents, because rodents can make their own essential fatty acids. How are we going to work with that within the models?

DR. BRUCKNER: Jeff, what are we going to do?

[Laughter.]

QUESTION: This is just a question. I don't have an answer. I'm just--

DR. BRUCKNER: Right, that's a good point.

DR. PREUSS: Before we have any other questions, let me just interrupt for a minute.

I was going to make a proposal right after this that given how early we are that we might like to simply proceed to the panel discussion and not break right now for lunch, but that way, we probably would finish an hour or two earlier than otherwise anticipated. I jump in now because I see some people starting to take an early lunch break.

Does anybody have any objection to going ahead now and just going on to the panel discussion?

[No response.]

DR. PREUSS: Okay; thank you. So that's what we'll do. So, after the questions are over, we'll take a five-minute break just so that they can set up the table here, and then, we'll go right into the panel discussion. Sorry.

QUESTION: Thank you. I'm Jennifer Sass with the Natural Resource Defense Council. Thanks for your talk, Dr. Bruckner.

Could you go to your conclusion slide quickly? I think it's one or two back.

DR. BRUCKNER: I wouldn't really call it a conclusion slide; just sort of a question slide. Let's see; I'm sorry. There we go.

QUESTION: Okay; would those statements be most relevant to the oral dosing, or would you also think based on your data and other data and your expertise that those would also be relevant to inhalation? To me, it seems the inhalation is

really different than the oral dosing.

DR. BRUCKNER: Right, they're not at all relevant to inhalation. Inhalation is, you know, directly into the systemic circulation.

QUESTION: And then into the blood; your own work, what you presented today, shows it from inhalation, it's getting very quickly into the blood circulation.

DR. BRUCKNER: Right.

QUESTION: And so, then, I guess my next question would be from an EPA health risk assessment type of perspective, we know from contaminants in the water, specifically VOCs like trichloroethylene, and I know with a lot of other chemicals, it's been shown that you can actually inhale a substantial amount, a significant amount, through the water in showers and spray and things like that. Showers are a big one.

What do you think about that? Do you have any knowledge of TCE in that area? Or have you considered that?

DR. BRUCKNER: I've looked at quite a bit

of the data in terms of relative contribution of inhalation and ingestion, and I guess my bottom line is that certainly inhalation contributes to the dose which would escape this mechanism. But how can I put this? I've seen varied results in terms of total contribution by inhalation. Of course, it depends upon a lot of variables in terms of how long, how hot the water is and those types of things.

But I think ingestion is maybe a little bit more important than inhalation, but inhalation and dermal, which also bypasses presystemic inhalation largely--

QUESTION: Right.

DR. BRUCKNER: --would be not as important but still an important contributor.

QUESTION: Then, that, I mean, again, from an EPA perspective, when someone is considering how your work is relevant to the health risk assessment, and clearly, it is, this doesn't really speak to the potential risk of inhalation of TCE from showers as a water contaminant; is that right?

DR. BRUCKNER: That is correct.

QUESTION: Thank you

DR. WANG: Jung Der Wang from National Taiwan University.

I just want to make two comments. One is just to supplement this lady's comment, because in our study in Taiwan, our people usually boiled the water. Usually, we boil the water before drinking, whether it is tap water or whether it is regular well water. So in our recent assessment, we actually conducted a study to try to boil and to see the residual tetrachloricity and perchloricity in the water left over after boiling. And we found that within 1 minute, everything almost was undetectable after boiling.

So our risk assessment actually considered the skin absorption and also inhalation from the shower and also from taking a bath, and we found that the risk, actually the potential risk for well water.

Number two, from your comment over here, you say that reaching extrahepatic organs. So

actually as you indicated that it should enter the portal vein and enter the liver. So cancer risk for liver may still be possible; I don't know for --

DR. BRUCKNER: Certainly, it goes to the liver, so the liver is not protected.

DR. PREUSS: Thank you. Okay; in that case, it's a quarter to. We'll take a five-minute break to set up the table, and then, we'll come back and have all of our speakers join us on stage.

[Recess.]

DR. PREUSS: I think we're ready to begin. if we could ask our speakers to come up front and everyone else to take their seats.

Okay; then, let me turn to the panel and you recall, I mentioned first thing this morning that I wanted you to think about one question, which was, what is it that you heard during the course of the two days that you would particularly call to EPA's attention? In other words, things that you found particularly important in terms of the assessment that we're going to do or things that perhaps you saw in a different light from what

you had seen previously or things like that?

So that's really the major part. As sort of a subquestion, I'd like to ask you number one, if any of you have some work underway that you didn't have a chance to mention that you think now becomes more relevant in light of the presentations that have gone on; maybe you could briefly speak about that.

And finally, if you think that there's a set of--not a set of--if there are some particularly important published articles that you have not heard mentioned here in the past two days that you, again, that you want to commend to our attention, that would be helpful.

And the reason why I say that is that the process that we are planning to follow now to complete our assessment of TCE is that we have proposed a two-step process with the National Academy of Sciences, the first step being that we would write a series of issue papers, a small set of issue papers that deal with some of the more controversial issues and bring those to the Academy

for discussion, to a panel that the Academy would appoint for discussion and get comments from them as to how they viewed the science and that, of course, other organizations and other people would be free to submit issue papers or comment on the issue paper situation we had written or anything like that; and then, secondly, that we would then take all of that information and then create a new assessment which, again, we would take back to the Academy for peer review and public comment and so on.

What we are trying to do is to try this as an experiment to make this an extremely transparent process so that everyone can see what the issues are, what the science concerns are, what the debates are about as we go along rather than just seeing them when we finish our document.

So with that as background and the understanding that the work that Jeff Fisher talked about regarding PBPK is going on as sort of a joint effort, I'd open the floor to all of you and please ask you to begin.

Dick, could I ask you to start?

DR. BULL: I didn't know you were going to ask me to start. I was just rapidly scanning. But there were some things that--I'm not sure I was completely unaware of them, but I didn't realize how far they had progressed.

And I think one of the things in trichloroethylene that you're really going to have to turn your attention to is this issue of intersusceptibility issues, I'd rather call it in terms of sensitivity but susceptibility issues. And I think that's brought out by Pumford's work on the immune responses stimulated by--that's quite interesting, and I think the doses are in the range that it may be one of the more sensitive things that I saw. I didn't really do the back of the envelope calculation, but it's certainly below the doses that are used in the cancer bioassays.

The issues of sensitive population probably plays out in a lot of different ways with trichloroethylene. Metabolism is clearly one. That's the one thing that I think we're learning

from the animal studies is--and maybe even from the human studies, although I'm not quite sure--we're seeing a tremendous a lot of inconsistency with trichloroethylene, and if you start trying to make sense out of the tumor data, you can't even make sense between mice and rats, because you're getting tumors in one species and not the other, and the question are some of the mechanisms really important or not in producing human tumors is another question but we don't have a real consistent database that you can point to this is really going to be a human health risk at some particular level of exposure. I mean, I don't think we're arguing about effects at high doses. We're talking about how you would deal with effects at low doses.

There are some things that did not come up. I've seen papers not only from my own lab but others that suggest that there are some things that maybe should be started to look at a little more in a more sophisticated way, in the sense we're still focused, I think, too much on the peroxisome

proliferation kind of thing. I think that occurs; we know it occurs; we have some notion of what the implications or lack of implications that might have for humans, but we know we're getting some other things that are going on with trichloroethylene that I think need to be looked at more seriously, and that was one of the reasons I was trying to point out the issues with dichloroacetic acid to the extent it plays a role. Clearly at the doses you're producing of trichloroethylene, it's not through a peroxisome proliferation type of mechanism.

I know of a paper that's out for review that should be very interesting when it comes out, and there's been work now published, that's being readied for publication, in which they've looked at a PPA or alpha knockout mouse and the tumor response to trichloroethylene and the two acid metabolites that I think you should keep an eye out for; probably some months before it's out, but it's coming along.

I'm not going to comment on the

epidemiology. I'll just make the epidemiologists mad.

[Laughter.]

DR. PREUSS: Okay; thank you.

Dr. Pereira?

DR. PEREIRA: Well, you brought up peroxisome proliferation. I'll talk a little bit about that. But first, I'll go back and remind the audience that, you know, in cancer, you're looking at oncogene expressors that are going up and tumor suppressor genes that are going down. In cancer prevention, which the other half of my laboratory work on, you're looking at the opposite effects. You're looking at agents that increase the expression of tumor suppressor genes and decrease the expression of oncogenes.

And with respect to that, the agents that NCI is looking at and other people are the peroxisome proliferators or actually the PPR gamma and alpha. These agents appear to be very good in preventing colon cancer. Both the PPA alpha and the PPA gamma, a lot of this has to do with the

fact that these receptors bind to RXR, and that is involved with the recruitment of histone deacetylase, and that all involves with the reactivation of tumor suppressor genes.

And also with the understanding that you don't want to do any harm in regulations, my question would be is there any consideration to the fact that these agents might actually prevent colon cancer, because the peroxisome proliferators, some of them, are probably the most potent chemopreventive agents for colon cancer in rats, and also, they're looked at with respect to chemotherapy, especially the gamma.

The glutazones are being looked at as adjutant therapy to get to people after they resection the colon, and I would wonder since DCA, TCA, trichloroethylene are good peroxisome proliferators, I would suspect and predict that they would probably be very good in preventing colon cancer in the models.

DR. PREUSS: Anyone else have an observation?

Yes, please.

DR. BRUCKNER: Just one other thing that we're looking at, which I didn't mention, is the question of, you know, the variability from one person to another in levels of 2E1, whether it be environmental or genetic, because there was a paper by Greg Kedderis about three or four years ago now, I think and a more recent paper in which they raised the question of if you have 2E1 in excess that can metabolize all of the trace levels of trichloroethylene you would expect, what difference does a tenfold variation make if the least of us is metabolizing all of it?

So in terms of human variability, in terms of oxidated metabolism, that's one of the things we're looking at.

DR. LASH: I just wanted to comment that when you had asked us to think about what the EPA should focus on, I thought about that in terms of some of what we're doing, and I guess two points that's been mentioned already are variability, to better, you know, I think there's increasing

documentation of that and what the implications are.

And the other issue which I think we found in our in vitro studies in human proximal tubular primary cell cultures is how critical doses in terms of the nature of the response, that actually, over different dose ranges, you get responses that really can range from almost protective, where you get maybe some degree of a certain type of injury and then repair and proliferation versus outright necrosis at higher doses or a much higher degree of apoptosis.

So I think you can extend that to in vivo, really, and try to consider really that at different dose ranges, and I guess everybody considers dose, but then, I think it sort of gets forgotten, in the sense that people will still, then, try to derive implications from studies at very high doses, and I think you can't directly do that because the very nature of the response can be very different at lower doses than at higher doses, so that's one thought I'd like to--

DR. PREUSS: Thank you.

Dr. Wang?

DR. WANG: Thank you.

In terms of susceptibility, I think I would like to comment on if, when a person is a viral hepatitis either B or C carrier, then, he is more likely, his liver is more likely to get inflamed if given a very small dose of these halogenated hydrocarbons or alcohol, et cetera. And I think that this would increase the likelihood of liver cancer, from our understanding, and I don't know whether this will be useful for your comment when you are writing the final draft.

DR. PREUSS: Thank you.

I'm sorry; I don't have good peripheral vision.

DR. BURCH: Just taking off on the last two comments that were made by the panelists, I would also like to emphasize some of their thoughts.

From my perspective, it seems as though there are a number of different endpoints that

should be considered, need to be considered, I'm sure will be considered in your evaluation. There was a real emphasis here on carcinogenesis. We also saw some very interesting talks on immunotoxicity and autoimmunity as well as the neurotoxicity or the neurobehavioral endpoints that I presented.

So the question becomes where do we see—at which endpoints de we see at the largest doses, you know? Dose becomes a very big issue.

What is seen first? Those effects are going to be the targets for protective measures, I would think, from a public health standpoint.

And also, this idea of sensitive populations; I mean, that can't be overemphasized. Certainly, the prevalence of alcohol consumption is very high. Other factors, including genetic differences or other differences such as hepatic infections, how they interact and modify the effects of solvent exposures can be also very important.

DR. PREUSS: Dr. Lacey?

DR. LACEY: Yes, I think regardless of endpoint, though, most of the population based attempts to get better risk estimates are going to suffer from what we saw in a lot of our data, low statistical power, whether it's a high dose or low dose of exposure.

One way to potentially address that would be to put more emphasis on exposure biomarkers, whether that's through some of the new protein-based techniques, genomic-based techniques, some modeling, perhaps, statistical modeling of exposure over time.

But it's clear that traditional epidemiologic approaches are going to have a tough time further refining risk, and so, there's going to need to be a much more concerted effort for translational approaches, I think, to get population based risk estimates.

DR. PREUSS: You think that that's the case in general or for particularly rare diseases such as you were talking about?

DR. LACEY: I think it becomes

increasingly important for rare diseases like scleroderma, like some of the cancer endpoints.

DR. BULL: Just to add to the issue about dose that Larry was bringing up, and I think this is a critical issue, and it goes to the comments that were being made relative to in interindividual susceptibility and the most sensitive endpoints.

If you have animal experiments that are showing things at gram per liter or hundreds of milligrams per liter in a particular area, one of the things I really take a little bit of umbrage at in a way, and I will be a little bit provocative here, is using that as a background for a biological plausibility for response seen in an open epidemiology experiment that is really dealing with very small doses.

I think if those effects are real, I'd like to find other--a lot more thorough looking for other possible causes. I don't at all doubt effects of ethanol of the sort we saw, but the interaction is difficult to rationalize based on what I know on what kind of doses are going to

produce, you know, more or less irreversible damage in the nervous system, mainly ototoxicity, I have to admit.

But somehow, the agency has got to come to grips with that, and I think that was one of the big difficulties that came out of our 2000 effort is you got to find some way of getting a controlled experiment coupled with--whether it's in humans or experimental animals, to make some sense out of those kinds of things, because I don't think it can be done otherwise.

DR. FISHER: I guess I'd like to reiterate the use of human data; though it's very important, it's difficult quantitatively looking at epidemiology studies. And in the last risk assessment, there were attempts to use the human data in a quantitative fashion, and there's one particular table that shows some of the human endpoints or the most sensitive endpoints based on epi data.

If you looked at some of the studies, you would see that the exposure site was unknown,

really, so I think to use the human epi data quantitatively, it's a tough way to go, and you should have info on exposure and information on dose-response, some existence of dose-response.

DR. GUENGERICH: I'd just like to bring up one thing again with regard to humans. And it concerns something, two of the metabolites that we've talked about the last couple of days, chloral and dichloroacetic acid, have been used as drugs. And I guess I'm sort of thinking out loud here wondering if information about those can be utilized.

I'm not familiar with all of the literature. I think one of the problems on those--it might be a problem is that chloral had been used as a sedative. It's not really, as I understand, chronically used. I think the uses of dichloroacetic acid are somewhat more chronic, and I don't really know how many people are involved with the use of these or even what kind of work went into the registration. These are terribly old drugs and may not have been analyzed; perhaps

somebody has some insight.

DR. PREUSS: Yes, Dr. Bruckner?

DR. BRUCKNER: I was just going to mention about chloral hydrate. The last I've looked, which was about six months ago, I think it's still probably the most widely-used pediatric, you know, sedative. And I guess I couldn't find any epidemiology studies that had ever been done. Of course, I know a lot of children have been dosed with chloral hydrate. Of course, it is usually in an acute situation, but I just wonder if that might be looked at and see if that's possible, because I don't know of another human population that would be better to study.

DR. BULL: Yes, the dichloroacetic acid thing is interesting, because it was originally proposed as an oral hypoglycemic agent, and its use in that area was abandoned fairly early. I don't think it ever got widely used.

But it is still used, but it's used in such an unusual circumstances. I mean, it's in these kids with hyperlacticacidosis, and it's

lifesaving in that case. But in point of fact, the doses they are getting to those kids are very similar to the doses that are producing cancer.

So I don't know if I'd want to take advantage of that particular population, but, I mean, clearly, if that could be, there are people that use it.

The other issue that, and I didn't want to get into any details on this, but the one issue that is really of interest with the dichloroacetic acid is its real rapid metabolism, especially as you get down to lower doses, where you're not seeing the suicide inhibition of the enzyme. And I don't know--I've talked to a guy at Anders, and they've looked at distributions of that isoform. Nothing seems to be too interesting in the sense that they're active towards dichloroacetic acid, but the level of activity is just a factor of, you know, a twofold, threefold difference.

But if you ever had a null, it might be interesting, particularly in younger individuals if the human development pattern follows similarly to

the rat, where, as I mentioned earlier, you no longer have an inhibitable enzyme in the late adult animal. So that could be an issue.

Now the late adult animal still metabolizes dichloroacetic acid very quickly, so there are a lot of little things like that, I think, that could be looked at fairly directly.

There are knockouts for the GST zeta as well. They have one at Oregon Hill Sciences Center that they're doing work with, mainly from the context of this group of kids with hyperlacticacidosis.

DR. PREUSS: Yes?

DR. PEREIRA: With the question of developing biomarkers for exposure, I think it is important to make a distinction between biomarkers of exposure and talking about biomarkers of risk or carcinogenic activity. A lot of it goes back to cystochromatidic strains and people have an increase; what does it mean? The same questions would be asked about cell proliferation and about the microarrays.

And a lot of this comes back, again, from the studies at NCI, and we're doing some of them, looking at in people as well as in animals for biomarkers for cancer prevention. Originally, it was looked at that you would look for agents that would alter cell proliferation; in other words, decrease it and then turned it out that cell proliferation had really nothing to do with the effect on cell proliferation as to whether or not an agent would be a chemopreventive agent.

Very similarly, if you look at cell proliferation going the other way with respect to whether it's going to cause cancer, it might be a decent marker for exposure assessment, but as a marker for whether there's a cancer risk or what it is, it's probably pretty poor.

DR. PREUSS: Dr. Hansen?

DR. HANSEN: Yes, I agree with you that the existing epi data can only be used in order to evaluate whether there is an increased risk of a certain cancer or other disease. I'm sure that the quality of existing data are not sufficient in

order to extrapolate from those doses we have seen in the occupational environment, for instance, into the environment in general.

So at least our studies can be used in order to evaluate is there a risk or isn't there a risk but not to dose-response.

DR. PEREIRA: Are you taking from the floor?

DR. PREUSS: Sure.

QUESTION: Ernie Blumen, Dow Chemical. That might be the appropriate time to inform you about three studies that are in the pipeline on human effects. One study is on metal degreasers, 70 metal degreasers that have been degreasing for up to 20 years, exposures for up to 250 ppm based on TCA measurements. And people looked at the indicators for kidney damage, like residual binding protein, NRG, albumin, and there were no indications of damage to the kidney.

But also interesting was that there were suggestions for the alternative explanation for kidney cancer in male rats to be operating. It's

the formate from excretion based on the inhibition of the folate cycle. And what was seen was that in humans at the higher levels, there was an increase in formate excretion and matziomalonic acid excretion. So this might be seen as supportive at least for the pathway to operate. Whether it causative, we are not sure, yet, of course.

This study will be published, will be available in two months, I think, because the galley proofs are already there.

Another study is a study done to confirm the findings from the Henschler study, a case control study of kidney cancer. Cases were set up in an area where there is a 20 percent prevalence of exposure to TCA, because there is a lot of metal degreasing going on. So 18 cases have been identified, and the analogy is taking place now in the cohort, in this group of cases, and we expect the results to be available at the end of this year, maybe beginning of next year.

In this group, we are also doing a VHL analysis. So we try to see if we could

independently confirm the findings by Brown. These results should also be available at the end of this year or the beginning of next year.

DR. PREUSS: Thank you very much.

Yes, sir?

QUESTION: Jay Pandey from Charleston.

I had a question from Dr. Pumford. You have shown the effect of TCE and antinuclear antibodies but which are common to all autoimmune diseases, virtually all autoimmune diseases. Have you looked at any disease-specific autoantibodies, diseases where TCE has been implicated, like SLE and scleroderma. Have those increased, and have you looked at it or not?

DR. PUMFORD: We have not looked at that. I understand what you are saying, though.

DR. PREUSS: I don't know if you have your hand up or oh, there. Thank you. Sorry.

QUESTION: Hi, Jennifer Sass with the Natural Resources Defense Council.

It's been a long day and a half for me. I've struggled to understand what's going on here,

and I just want to ask a kind of a question that puts some stuff into perspective for me.

First of all, I guess, with the Danish epidemiology studies, there have been two epidemiology studies, and my understanding of the presentations yesterday and from the informative questions afterwards is that there were two different cohorts, one, the first study that was published in 2001, and then, the second that was published in 2003.

So my first question to the author, Dr. Hansen is both--my understanding is that both of those studies showed an excess in esophageal and also in the non-Hodgkins lymphoma. Is that right, or am I getting mixed up?

DR. HANSEN: Yes, that is correct.

QUESTION: And although the numbers are different, one of the reasons why is that the cohorts are different, and in the more recent study, the 2001, my understanding now from short conversations with you is that you think that although it does show an excess, you are confident,

that it is possible that that may have underestimated the risk, because the assumptions built into the model assume that everybody in the plant was exposed when, in fact, that probably isn't the case; is that correct? Is my understanding correct?

DR. HANSEN: Yes.

QUESTION: And then, the interesting thing from all of the animal studies that have been discussed and some of the metabolic studies and even the studies of the modeling is that I didn't see those cancer types being addressed in the animal or in vitro work.

So I wonder if some of the authors can speak to that, or did I miss it? Mostly I saw liver being addressed and some kidney.

DR. BULL: For the large part, that's what shows up in the animal studies, both the trichloroethylene and the metabolites to the extent they've been looked at. So it's clear that you can produce at least liver tumors with the metabolites. Trichloroethylene itself is not particularly

effective in the rat as a liver carcinogen. It produces--the kidney cancers are at low risk.

The esophageal cancers, to me, when I think of esophageal cancers, I start thinking nitrosamines, so I mean it's kind of one of the things that I worry about in an industrial situation where nitrosamines, they're so much more potent than trichloroethylene as a carcinogen, I start worrying, what's the, you know, or maybe they ate too much bacon for breakfast or whatever, because your effect doses are--

DR. GUENGERICH: And tobacco is another.

DR. BULL: Yes, and tobacco is not a low dose particularly, but you see esophageal cancers with--I'm not sure. Outside of the nitrosamine group, do you know off the top of your head? I don't know. I can't say that, but as soon as I--that was a very interesting finding. I just don't know what to make of it.

DR. HANSEN: But no one else has found it.

DR. BULL: That's true, too.

QUESTION: He doesn't believe that non-Hodgkins

lymphoma has ever been seen in animals.

Right. So there's no animal model, although there was some autoimmune type discussion that I thought was interesting and potentially relevant someday.

My other question is I wonder if the epidemiologists and the animal modelists could have a short discussion that might help me about some of the differences between either chronic sustained exposures or peak exposures, because I notice that's one thing that came up in the epidemiology is that there might seem to be more relevance with looking at peak exposures rather than long-term. Correct me if I'm wrong. And I know with formaldehyde, that came up recently in the NIOSH study, and then, in looking back at the recent NCI study that's coming out soon is that when peak exposures were considered, there was actually a correlation with the exposure to formaldehyde, whereas, when they looked at the average or the chronic, there wasn't.

I wonder if you might comment on that.

DR. FISHER: From a modeling perspective,

I could comment. It's very easily--to use that as a dose metric, peak levels, and you can use that information just like area under the curve, and you can have several dose metrics to compare with effects.

QUESTION: I wonder if you think that might be worth looking at.

DR. FISHER: Yes, we're planning on using a concentration as a dose metric or peak, as you're calling it.

QUESTION: And I wonder if the epidemiologists might comment about whether you think that's something that might be looked at more closely in the data and that some of the studies that aren't looking at that might possibly miss effects or not.

DR. PESCH: Many of these epidemiological studies suffer from missing measurements, so if you do not have measurements, you cannot reconstruct the exposure metrics.

And so, on the one side, it is supported by the metabolism that maybe peak exposures are of

a higher importance. But on the other hand, workers who have peak exposures also have a long term exposure. So it's hard to disentangle which exposure metric would better fit the data.

In our data from the MURC study, we could not see a dose-response effect with cumulative measures, but all risk estimates were very low. So it's not a clear answer to your question.

DR. BULL: I could add a hypothetical or semihypothetical to that in the sense that I think that's one reason the Henschler and subsequent studies kind of keeps peaking is because if you--and it's perfectly consistent with the animal data. If you expect that the individuals need to get to a cytotoxic dose of trichloroethylene to the kidney, that kind of fits with what the animal data say. But you really have to get to very high doses, and it's probably not a low dose phenomenon, and I think that's what comes out of the rest of the studies.

So certainly, peak exposures can be important if they get high enough, get over the

threshold you need to produce significant cytotoxicities. So I'm putting words into other people's mouths, but I think that's the reason some of the investigators that have been interested in that particular pathway have really pushed that particular finding for some period of time, because it makes a certain amount of sense.

DR. PREUSS: Dr. Lacey?

QUESTION: Again, I just want to support what Dr. Pesch said, that I've been trying with other chemicals including benzene to disentangle high exposures, cumulative dose, and peak exposure, but I correlate that it's very difficult to get them separated out, and although intuitively it might appeal to people, epidemiology, I think, is not good enough to get those things, those effects.

DR. LACEY: I am glad that the word threshold came up, because on the assumption that any individual peak exposure reaches a threshold and then initiates with some certainty a disease process, be it cancer or scleroderma or any other condition, the case control design in particular is

very well suited to identifying those associations.

So I would argue that if very high peak exposures were strongly associated with, I think, for example, scleroderma, we, I think, would have seen it in our data. And so, to date, the absence of those associations, those types of associations, leads me to think that, in fact, peak exposures are more important for the conditions we talked about as a contributor to long-term exposure or as a marker of long-term exposure. But again, that's based on data with the limitations that we've been discussing.

DR. PREUSS: Larry?

DR. LASH: I just wanted to add that I sometimes have been troubled when I see --when they talk about peak exposures and then cumulative doses, because I think you have to just as a basic principle that when you consider a compound like trichloroethylene as compared to, say, something like dioxins, PCBs or, say, cadmium, which have very, you know, long half lives, that the consideration of cumulative dose is not the same

for something like TCE that's metabolized and excreted.

And true, there's some storage in fat, but it's not the same type. So I think it goes along with the threshold concept that cumulative dose may only be important when you get past a certain point, and I think it's--you know it's something important to consider.

DR. PREUSS: Dr. Wang?

DR. WANG: To respond to this, we should consider--it's also published--because the underground water, we probably could not--we probably should not only consider one particular, like a TCE exposure, because for the underground condition, the anaerobic reductive dehalogenation may produce a mixture of exposure of halogenated hydrocarbons.

And what we did in ICR mice, that we used a dose which is about less than one milligram--we used a micrograms per cc and feeding chronically, feeding all these ICR mice for 16 and 18 months and 18 months, and the male showed an increased frequency

of hepatocellular adenoma, and the female, we have increased mammary cancer, mammary adenocarcinoma.

And I hope that this probably can also explain some of the discrepancies that --

QUESTION: I just had a question for Dr. Lacey. As you well know, your results are at odds with several other studies which have shown a significant association between TCE and scleroderma. Is it something genetically different about people from Michigan and Ohio? Can you comment on that?

[Laughter.]

DR. LACEY: They're at odds on the issue of statistical significance, which I think arises because of the low exposure prevalence. But as we concluded in the paper, we thought that the magnitude and the direction of the association was consistent with others. We just couldn't rule out chance.

DR. PREUSS: One more?

QUESTION: Yes, hi, Laura Green, Cambridge Environmental and MIT.

I have a question for Dr. Guengerich as the chemist on the panel and also Dr. Pesch and some of the epidemiologists interested in kidney cancer.

My colleagues and I and others have hypothesized that the Henschler cluster of kidney cancer could be due not to TCE but to dichloroacetylene. The unusual thing about cardboard making is there's a lot of lye vats around, and the dehydrohalogenation catalyzed by base of TCE is an, I believe, well-known phenomenon and the animal toxicologists on the panel probably know that dichloroacetelyne is an extremely potent nephrotoxin and nephrocarcinogen in both rats and mice, both males and females, at, you know, much, I mean, it's a striking nephrotoxin and nephrocarcinogen across species.

So I guess I'm wondering from the chemical and maybe from the epidemiologic and maybe Dr. Cherrie from the exposure point of view, whether separate from TCE, whether the perhaps the unique formation of dichloroacetylene in the cardboard

environment, given the lye vats, might be a reason that the results are so disparate from one factory to another.

DR. GUENGERICH: Well, I can start, and I don't really have an answer about that, because, well, it gets into contamination. You know, you can raise several issues over the years with some of the animal studies that have been done with contaminations with epichlorohydrine and epoxybutane and things like that.

I guess I can't, you know, it's conceivable that this might be a factor, although I guess I'm not in a place to know if it really contributes, and I guess, basically, one would actually have to have real chemical data on the presence of the dichloroacetylene in the industrial settings.

Perhaps somebody else can expound on that.

DR. CHERRIE: Well, again, I think I'm not the right person to answer the question, but I think it's something that could be investigated experimentally to see whether--there's always the

possibility in that situation for dichloroacetylene to be produced.

DR. PREUSS: No other questions from the floor, and I don't see hands being raised on the panel.

I'd like to thank all of you for joining us and participating with us. We at EPA very much welcome the information that you've brought. And so, we thank you very much, and I'd like to thank the audience for their good humor and their patience. Thank you for having joined us also. Good afternoon.

[Applause.]

[Whereupon, at 12:39 p.m., the meeting concluded.]