IOWA CONFERENCE ON

EMERGING ENVIRONMENTAL HEALTH ISSUES

- Drinking Water Disinfection By-Products
- Synthetic Organic Chemicals and Birth Defects
- Endocrine Disrupting Chemicals

September 23-24, 1997

Hosted by: The Center for Health Effects of Environmental Contamination (CHEEC)

THE UNIVERSITY OF IOWA

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Preface

The ***Iowa Conference on Emerging Environmental Health Issues*** was held on the campus of the University of Iowa on September 23-24, 1997. The conference was co-sponsored by the Agency for Toxic Substances and Disease Registry, the Roy J. Carver Charitable Trust, the Robert L. Morris Memorial Fund, the University of Iowa Hygienic Laboratory, and the Center for Health Effects of Environmental Contamination (CHEEC) at the University of Iowa. The conference was one of a series of conferences, workshops and other educational forums held during 1997 to celebrate the 10th anniversary of the Iowa Groundwater Protection Act, landmark legislation that established a wide variety of water programs in Iowa.

Conference speakers discussed ongoing research, current policy issues, and potential prevention and control measures on three emerging environmental health topics including drinking water disinfection by-products, synthetic organic chemicals and birth defects, and endocrine disrupting chemicals. Conference goals were to educate the public, public health professionals, environmental scientists, and water industry professionals on the utility of environmental health research by demonstrating potential impacts on public health in Iowa; to establish the importance of interdisciplinary research, collaboration and communication by identifying and addressing emerging environmental health concerns, with the goal of defining prevention programs and control strategies; and to showcase the leadership and collaborative role Iowa institutions have played in conducting and assessing research on emerging environmental health issues.

Special thanks to Dr. George Hallberg, Dr. Jeff Murray, Dr. Charles Lynch, Dr. James Hanson, Dr. Gene Parkin and David Riley for their efforts in planning the conference, contacting speakers, and moderating the program. Thanks to Carrie Kaiser-Wacker for her help in planning and facilitating the event. Finally, very special thanks to my co-Editors on these proceedings: David Riley and Samantha Van Nyhuis.

Peter Weyer
Co-Editor
Program Coordinator, CHEEC

Welcoming Address

Gene Parkin, Ph.D., Director, Center for Health Effects of Environmental Contamination, University of Iowa

I would like to welcome you to the *Iowa Conference on Emerging Environmental Health Issues*. We have a very interesting program planned for you. This conference is part of a year-long series of activities celebrating the 10th anniversary of the passage of the Iowa Groundwater Protection Act in 1987. To commemorate this 10th anniversary, Governor Branstad has proclaimed 1997 the *Year of Water* in Iowa. CHEEC was established by the Groundwater Protection Act along with our sister research centers, the Leopold Center for Sustainable Agriculture at Iowa State University, and the Iowa Waste Reduction Center at the University of Northern Iowa.

I have a couple of announcements to make. First, there would be no Center for Health Effects of Environmental Contamination if it weren't for the efforts of three people, two of whom are in the audience today. These three gentlemen were primarily involved in the creation of CHEEC and its inclusion in the Groundwater Protection Act. We like to call them the founding fathers of CHEEC - they are Dr. William Hausler who is Director Emeritus of the University of Iowa Hygienic Laboratory, Professor James Hanson who is in the Department of Pediatrics at the University of Iowa, and Dr. Peter Isaacson, who couldn't be with us today, who is Emeritus Professor in the UI Department of Preventive Medicine and Environmental Health. Peter served as the first Director of CHEEC when we started back in 1987. We owe a great debt of gratitude to those three gentlemen.

I'd also like to acknowledge the efforts of three other people, without whose efforts we wouldn't be having this conference today. They are primarily responsible for writing the proposals that helped fund this conference, organizing it, selecting the topics, getting the speakers and making the conference a success. They are: Peter Weyer, the Program Coordinator of CHEEC, David Riley, a Program Assistant with CHEEC and Dr. George Hallberg, the Associate Director of the University of Iowa Hygienic Laboratory. Thank you gentlemen.

I should also mention the sponsors of this conference in addition to CHEEC. They are the Agency for Toxic Substances and Disease Registry, the Roy J. Carver Charitable Trust, the Robert L. Morris Memorial Fund and the University of Iowa Hygienic Laboratory.

I would like to introduce Dr. David Skorton, Vice President for Research at the University of Iowa. He also serves as a special assistant to the President for the University of Iowa Health Sciences Center. He's been here for 17 years and holds joint appointments in the Departments of Internal Medicine and Electrical and Computer Engineering. Under Dr. Skorton's excellent leadership for the past several years, the University has set records for generating external research funds. Dr. Skorton has always been very supportive of our activities in CHEEC.

Welcoming Address

David Skorton, M.D., Vice President for Research, University of Iowa

It's a great pleasure to be here today. I do open a lot of conferences and I always wonder why you have University officials open these conferences; I suppose it's for three reasons. One is to let you know that the University as a central entity views the particular conference as important. I'm going to get back to that one in a moment. Secondly, to tell you a little bit about how the University is doing and thirdly to welcome you. So let me take them in reverse order. I do want to welcome you to the University of Iowa; those of you who are not from here or who have never been here or have been away for a while. In that regard I want to welcome back Professor Jim Hanson; he's a valuable member of the community who has been away in Washington D.C. and is now happily back with us. I also want to have a chance to welcome Bill Hausler, who is the guiding light and glue that held together the University Hygienic Laboratory. The Public Health Laboratory in our State is part of the University - it's sort of an interesting arrangement. Bill and the really superb staff of the Hygienic Laboratory have served two masters for a long time very well.

Let me tell you a bit about how the University is doing in terms of research and why the kind of investigation and application of knowledge that you're talking about today really fits in with trends in the University itself. The University has just had another record year in external funding - $212 million - keeping us consistently in the top twenty public research universities in the country, and nearly the smallest of the public universities in that group. We think per faculty and staff capita that it's a very strong institution. Where are the areas of growth in that $212 million? The two areas of growth that are the most identifiable are corporate sponsored research, research contracts with industries that are down-sizing, and also inter-disciplinary fields like those represented by CHEEC. Professor Parkin mentioned that CHEEC, as well as the Leopold Center, was established by the 1987 Groundwater Protection Act. Hearing that, you might wonder whether these are state bureaucracies set up in some parity system between the universities, sort of an entitlement program. I'm here to tell you that they're anything but that. These are extremely valuable centers. I know CHEEC the best because we have a special relationship with CHEEC in our office, as we do with the Hygienic Laboratory. CHEEC, the UHL and other environmental science areas on campus are not only very strong purveyors of public service in Iowa and the surrounding region, but are developers of research themselves, developers and discoverers of new knowledge. CHEEC, through the databases that they collect and manage, assists and fosters new knowledge development in other centers. That service, education if you will, through the databases and research, really makes CHEEC sort of a microcosm of the University's mission. I mean that sincerely.

Finally, what is it about this conference that would make me particularly happy to be here and why do I have appointments in these two seemingly different fields? There actually are a lot of people in cardiovascular sciences who have engineering backgrounds or engineering interests because of medical devices, so I'm not so odd in that regard. I will say that these backgrounds make me particularly sensitive and keen on interdisciplinary efforts. This is one of the most broadly interdisciplinary conferences that we will have on our campus this year, as you can see from the section of the brochure describing the areas that are going to be addressed; research, current policy issues, control and prevention measures in three emerging environmental health topics: water disinfection by-products, organic chemicals and birth defects, and endocrine disrupting chemicals. Just as in my background, this center and the issues you will be discussing are also at the intersection between environmental engineering and the health and biological sciences. I applaud your efforts to do this. I wish I could stay with you to enjoy it and I trust and am confident that you're going to have a fantastic conference.

Session 1: Drinking Water Disinfection By-Products

Epidemiologic research on cancer risk and chlorination by-products in drinking water

Kenneth P. Cantor, Ph.D., National Cancer Institute

Dr. Cantor is an epidemiologist with the Division of Cancer Epidemiology and Genetics at the National Cancer Institute, where he has directed studies of cancer and environmental factors since 1977. He has a major interest in investigating the relationship between cancer and exposure to a variety of drinking water contaminants, including chlorination by-products, nitrate, and arsenic. He has served as an advisor to National Academy of Sciences committees on drinking water, and to expert drinking water and health panels convened by the Department of Health and Human Services, EPA, and the World Health Organization. Dr. Cantor received a Ph.D. in biophysics from the University of California at Berkeley and an M.P.H. from the Harvard School of Public Health.

(Editors Note: Reprinted from 'Cancer Causes and Control'; Vol. 8, 1997. Drinking Water and Cancer)

Chlorination by-products in drinking water were discovered in 1974, and epidemiologic assessment of cancer risk started shortly thereafter. The by-product mixture results from interaction of chlorine with naturally occurring humic and fulvic acids from decomposed plant matter, and other organic chemicals. Hundreds of halogenated chemical species have been identified, including trihalomethanes, other haloalkanes, haloalkenes, haloacetic acids, other haloacids, halonitriles, haloketones, haloaldehydes, and others. Formation occurs in the treatment plant and usually continues in the distribution system.

The major determinant of by-product concentration is the level of organic precursors in the source water. This is the basis for most exposure estimates in epidemiologic studies, because precursor compounds occur at much higher concentrations in surface waters (lakes, rivers, reservoirs, etc.) than in groundwaters (wells, springs). In the absence of historical data on chlorination by-product levels, estimates of past exposure have been based on historical information about water sources, with the knowledge that surface water users likely had much greater exposure to chlorination by-products than consumers of groundwater. In many ecologic studies, and in most case-control studies based on death certificates, exposure was defined by the type of residential water source at death. Exposure in several case-control interview studies was estimated by duration of residence served by chlorinated surface water. Ingestion is the major route of exposure for the non-volatile by-products. Of equal or greater importance for the volatiles are inhalation and dermal exposures to compounds released from water, for example during showering. A related exposure issue is the apparent increase in trihalomethane formation when water is heated for domestic use.

It is not known which chemicals or combinations in the mixture may pose a carcinogenic threat. Differences in the chemistry of source waters and treatment practices influence the relative concentrations of mixture constituents. For example, the brominated by-products vary from place to place, depending on source water levels of bromide; and pH differences modify relative concentrations. Among classes of by-products, the most commonly found are the trihalomethanes (THM), with chloroform occurring at the highest concentrations. Haloacetates, the next most common, are receiving increasing attention. The THM in surface waters range from 30 to 100 μg/l or higher and treated groundwaters from 1 to 10 μg/l. THM levels generally correlate well with concentrations of the overall mixture, and they have served as surrogates of exposure. However, geographic variation in mixture constituents may also influence risk. Two THMs are carcinogenic in animal models as are several haloacetates, and concentrates of the higher molecular weight, non-volatile, by-products are mutagenic in bacterial systems. A chlorinated hydrofuranone is a potent mutagen.

A monograph from IARC reviewed the epidemiologic literature on chlorinated drinking water and cancer prior to 1990. The first epidemiologic studies were ecologic, and correlated age-adjusted, sex-and race-specific regional (usually city or county) cancer mortality rates with 1) surface as compared with ground source, and chlorinated compared with non-chlorinated water, 2) the Mississippi River compared with other sources, or 3) the level of trihalomethanes in the water supply. Incidence rates were correlated with water supply characteristics in Iowa towns, Norwegian municipalities, communities in Valencia, Spain, and Finnish cities. Bladder, colon, and rectum were the

sites of cancer most frequently associated with surface water, THMs, or chlorination status, or in the case of the Finnish studies, estimates of past water mutagenicity.

A second tier of studies took a case-control approach, using mortality records to identify cases and comparison subjects. Exposure variables in early studies in this group were characteristics of the water supply that served the decedent's residence at death, as listed on mortality records (surface/ground, chlorinated non-chlorinated, Mississippi River/other sources). Later studies used information about previous residences and sources of drinking water, inferred from the place of birth listed on the death certificate, or obtained from retirement system records or from next of kin interviews. These case-control studies focused largely on cancers of the bladder, colon, or rectum, and were generally supportive of positive findings from the earlier ecologic evaluations. An exception was a study in New York State that found no association for colorectal cancer with type of water source or imputed past THM level over the 20 years prior to death. Brain cancer was included in two studies, with suggestive associations. Most death certificate-based case-control studies were limited by a lack of information on potentially confounding risk factors, and by using the water source serving the residence at death as representing much earlier exposures. In addition, bladder, colon, and rectal cancer patients enjoy relatively good survival, and patients who die may be a biased sample of all incident cases, due to differential access to medical care or other factors. To summarize, case-control studies based on death certificates served to strengthen the hypothesis of a link, but their limitations preclude a stronger interpretation.

Case-control interview studies of incident cancer have been conducted in North Carolina, Wisconsin, Iowa, Colorado, Ontario, Maryland, and ten places in the United States. Cancer of the urinary bladder was the focus of five studies, colon of four, rectum of two, pancreas of two, and brain and kidney, one study each. Six anatomic sites were studied in Iowa and three in Ontario. In all studies, individual histories of water source and chlorine disinfection were developed by combining residential information from the questionnaire with historical data from water utilities. The time period encompassed by this history varied, covering the full lifetime of subjects in some studies, and shorter periods in others (for example, starting at age 20 or in 1940). In most studies, duration of chlorinated surface water consumption was used as a surrogate of long-term exposure to chlorination by-products. In addition, some authors estimated past exposure to THM (as representing the full mixture of by-products) by modeling water supply characteristics and recent measurements of these compounds. In addition to the case-control studies, incidence of bladder, kidney, and liver cancers, and mortality from all major cancers were evaluated in a cohort study from Washington County, Maryland, in which the exposure measure was the water source in 1963.

Findings from the colon and rectal cancer case-control studies lack consistency. Among the four investigations of incident colon cancer, the studies from North Carolina and Ontario observed positive associations with duration of chlorinated surface water exposure (in North Carolina among elderly cases only), and results from Wisconsin and Iowa found no association. The findings from North Carolina and Wisconsin are difficult to interpret because methods and results were only briefly described in the former study, and response rates were below 50 percent in the latter. Rectal cancer risk in Iowa was positively and consistently associated with exposure duration, whereas in Ontario, no association was noted. In studies with positive associations, odds ratios (OR) increased to levels of about 2.0 for the longest-exposed groups. The source of these apparent inconsistencies is unclear. If the positive findings were not spurious, the variation in observed risks for colon and rectal cancers may be due to geographic differences in the composition of by-product mixtures.

Bladder cancer findings are more consistent, with positive associations found overall, or in major subgroups, in five case-control studies and one cohort study. All were adjusted for cigarette smoking. Data from the National Bladder Cancer Study, conducted in ten locations in the United States, revealed increased risk with tap water intake, with the strongest dose-related risk gradients with intake among persons having 40 or more years exposure to chlorinated surface water. In Colorado, risk was associated with duration of residence at a chlorinated drinking water source, with OR increasing to 1.8 for 30+ years exposure (P trend=0.0007). In Ontario, bladder cancer risk increased with duration of chlorinated surface water use, and with estimated average THM level (OR=1.6 for 30+ years of 75+ µg/l THM). In Iowa, risk increased monotonically with duration of chlorinated surface water use (OR=1.5 for 60+ years exposure). Elevated bladder cancer risk among consumers of chlorinated water was also observed in the Washington County, Maryland cohort; however,

exposure was determined cross-sectionally at the 1963 interview, and the number of cases was small, providing but limited statistical power. A subsequent nested case-control study of bladder cancer in Washington County found an association with duration of residence with chlorinated surface water. Cigarette smoking appeared to enhance the risk posed by chlorination by-products in the case-control studies from Iowa and from Washington County. A bladder cancer case-control study from a population in western New York State mostly exposed to chlorinated surface water found a dose-response relationship with tap water consumption level. Water source and/or by-product level was not explicitly evaluated. The evidence bearing on the related issue of fluid intake and bladder cancer risk is equivocal. Increased consumption has been associated with a positive trend in risk and with no excess risk.

Pancreas cancer risk was evaluated in a nested case-control study within the Washington County cohort, and in Iowa, with inconsistent results. In Washington County, the OR was 2.2 for living in a residence served by a chlorinated surface source in 1975, as compared to having another source of water in 1975, usually a non-chlorinated private well. No association for pancreas cancer was found in Iowa, where lifetime exposures were estimated for cases and controls. Incident brain cancer risk was studied in relation to chlorinated water only in the six-site study from Iowa. Among men, but not women, there was an association between risk and duration of chlorinated surface water use, which was enhanced among above-median-level tap water consumers. Incident kidney cancer showed no evidence of an association, either in the Iowa case-control study, or in the Washington County cohort.

In summary, the evidence for carcinogenicity of chlorination by-products is strongest for bladder cancer, where associations were found overall or in major subgroups in five case-control studies and one population cohort study. Elevated risk of either colon or rectal cancer was also observed in a few well conducted studies, but results are not consistent, possibly due to geographical differences in the composition of the by-product mixture. Brain cancer incidence was elevated in the one case-control study that evaluated chlorination by-products. These results should be regarded with concern. They warrant further study in additional populations that include elaboration of by-product mixture chemistry and characteristics of individuals that may enhance risk.

Epidemiologic research on reproductive outcomes and disinfection by-products

Michele Lynberg, Ph.D., National Center for Environmental Health

Dr. Lynberg is an epidemiologist at the National Center for Environmental Health, CDC, where she has worked in the Division of Birth Defects and Developmental Disabilities since 1988. She is the Principal Investigator of the Metropolitan Atlanta Birth Defects Risk Factors Surveillance Project. She has a Ph.D. in epidemiology from the University of Iowa, an M.P.H. from UCLA, and she completed a fellowship in occupational and environmental health at the Johns Hopkins School of Public Health. Dr. Lynberg began her research in drinking water contaminants while at Iowa; her current research involves evaluating disinfection by-products in metropolitan Atlanta and their potential association with birth defects.

I'm going to review the three main categories of disinfection by-products. Trihalomethanes (THMs) have received the most attention because they're the most readily available by-product to study. They are regularly tested for and evaluated in individual water distribution systems. Basically, we're opportunistic when we evaluate them. The most commonly studied is chloroform, which occurs most frequently across the United States. Bromoform is more common in coastal areas; it's considered to be the more toxic of the THMs.

The next category is haloacetic acids (HAAs), which are considered to be more toxic as a category than THMs. They are not as frequently evaluated in water treatment distribution systems, so they're not as readily available for us to capitalize on. Then there's a whole host of other complex substances that occur with much less frequency, which we don't really know very much about. The point is that studies of disinfection by-products are almost universally studies of THMs. Epidemiologists consider THMs to be a surrogate measure of exposure; we're not certain whether that's the actual agent we need to be concerned about, but it's what we have the most data on, so it's what we use.

A number of studies have suggested associations between adverse reproductive outcomes and disinfection by-products. These studies have used a variety of designs. Ken Cantor went over the different types of study designs available to

epidemiologists. All these studies used existing water treatment data that were not collected for the purposes of doing studies of human health outcomes. I want to clarify what these differences mean for our studies, in which we are attempting to evaluate human exposure assessment.

The purpose of human exposure assessment is to study potential links between exposure and health outcomes. We generally rely on data that are used for compliance monitoring purposes. Those data are collected to protect the public from possible contaminants in drinking water supplies. In order to do human exposure assessment, we need a well defined, characterized population which is linked to exposure by location and time. The population relevant for compliance monitoring is all people all the time. The sampling schemes are quite different. We need enough data points to quantify actual exposure over time for an individual. I have a much easier job doing this when I'm looking at reproductive outcomes, than Ken does for cancer outcomes. It's a much shorter latent period - nine months of pregnancy versus a lifetime - it's a little easier to quantify. With compliance monitoring, you generally need to know where the high values are, where the problem areas are and what the problem times are. We need actual lab values. For example, what was the actual level of chloroform in a particular drinking water source that a pregnant mom might be exposed to? Compliance monitoring is conducted to determine whether violations occurred. We need to know a lot more when we're trying to link exposure to outcome; we need to know what's going on in that mom's residence. This has an enormous impact on where we are epidemiologically, with respect to what data are available to us. Both of these are very worthwhile efforts, and sometimes they can work well together. This information was taken from a recent article in *Environmental Health Perspectives*, and represents the bulk of studies on reproductive outcomes and disinfection by-products. I want to take a few minutes to focus on a couple of points.

In general, the associations are relatively weak. Ken talked about relative risk, I'm going to elaborate a little bit. What does it mean when we say we found a relative risk of 1.5 with a confidence interval of 1.2 - 2.1? Basically, it means that if we did a hundred studies, we would expect 95% of them to have a relative risk somewhere in this range. Often, it's interpreted if the confidence interval includes one, that any increase in relative risk may be likely due to chance. Some of these confidence intervals include one, for example, the Massachusetts study. When we're looking at total birth defects, the relative risks are the same, but the confidence interval in the New Jersey study excludes one while the confidence interval in the Massachusetts study does not exclude one. That's basically related to the size of the population and the relative power, which may weaken our ability to determine if there's a true association or not. Consistency between relative risks is important in the New Jersey and Massachusetts studies, which are the only two studies that have done any birth defects evaluations. The New Jersey study is the only one that's completed a birth defects study, because they are the only ones that have been able to look at individual defects. In the past, we have tended to look at birth defects as one entity. That is like considering cancer as one entity, it's inappropriate, they're really considering apples and oranges. We really need to look at individual categories of defects. I want to focus here on neural tube defects.

There was a relative risk of three in the New Jersey study, which excluded one, and that's important. Neural tube defects are one of our most commonly occurring birth defects. If in fact there is an association between disinfection by-products and neural tube defects, it would translate to a huge public health prevention potential. Developmental disorders, specifically birth weight less than 2500 grams, has a consistent relationship across the three studies that have been completed. While not statistically significant, a minimal increase of 1.3 in the background risk, if it were a true association, would still translate to potential public health significance when we consider all the women that are exposed. The other outcome I'd like to focus on is pre-term delivery. In most studies, the relative risks are right around one, and the confidence intervals include one. It looks like there's nothing particularly impressive going on, at least in the studies to date. There is an interesting difference in the risk of stillbirth between the New Jersey and Massachusetts studies, but the numbers are quite small and it's difficult to say what's going on.

The studies we've just discussed had fairly normal THM exposure levels, levels that we would expect to see based on the current standard. These are not levels that are extreme by any sense of the imagination. In the Iowa and New Jersey studies, the main THM was chloroform. I mentioned that the brominated THMs may be more toxic, but the fact is that chloroform occurs more commonly. If these associations are true, it's important to consider the potential for public health impact. The associations reported in these studies were modest for the most part; they were difficult to interpret because of small

numbers. The case definition was troublesome, with respect to the birth defects studies, and exposure classification has its inherent weaknesses. We need additional studies to determine whether these risks can be confirmed; it is important for risk-based decision making.

What additional research do we need? Based on what we've seen in the past, we need an interdisciplinary approach, with scientists from a number of backgrounds bringing their expertise to the field so we can, in fact, move the science forward. Studies need to include both adequate outcome information and adequate exposure information, and should be based on existing population-based studies of adverse reproductive outcome. They should also include information on other risk factors and potential confounders. In particular, they need to refine exposure classification. There are a few ways that could be done, including predicting exposure at the point of consumption, such as at the mom's tap, rather than in the distribution system. We need to look at temporal and spatial-specific exposure assessment for other disinfection by-products besides THMs. We need to potentially model and validate our modeling efforts by taking specific measurements, and we need to attempt to evaluate biologic levels of disinfection by-products in blood and urine.

We're trying to do some of this in metropolitan Atlanta. Our goal is to provide additional information on the potential link between DBPs and birth defects in both a timely and cost efficient manner. We have two important efforts going on which piggyback together and provide a strong framework for our approach. The most significant one is the Metropolitan Atlanta Congenital Defects Program, which has been in existence since 1968, and gathers information on all births in the metropolitan Atlanta area. We currently have close to 900,000 births evaluated, which includes almost 30,000 infants with birth defects in our Registry. We have a substantial potential to evaluate specific birth defects. As you recall, that's been one of the problems that's plagued birth defects epidemiology in the past. Researchers don't generally have the luxury of 1,000 cases of neural tube defects to evaluate. We also have 2,600 CNS defects and infants included in our Registry, and a substantial number of cardiac defects and oral clefts. These represent the bulk of the birth defects that occur in our population. Piggybacked onto the Metropolitan Atlanta Congenital Defects Program is the Atlanta Birth Defects Risk Factor Surveillance Project, for which I'm Principal Investigator. This project began in January, 1993. It includes information from interviews of moms, and we also collect biologic samples on both the mother and the infant. We annually collect about 300 interviews with mothers of cases and 100 interviews with controls. As of August 1997, we have about 900 cases and 500 controls interviewed; we have biologics on about half of those.

What we'd like to do with the Birth Defects Risk Factor Surveillance effort is build on the Centers for Birth Defects Research and Prevention, of which Iowa is one. There are currently eight cooperating sites in that group, which is the largest collaborative effort ever undertaken on birth defects research. It has a number of strengths and opportunities. The most important for our purposes are the diverse water supplies that will be available and the potential for looking at biologic markers of exposure. In all these sites we use the same maternal interview to collect information on everything possible with respect to factors important to the occurrence of birth defects.

Back to metropolitan Atlanta, and what we're doing now. In the five county metropolitan Atlanta area, we have six water treatment systems which provide the framework for the study that I'm working on with Dr. Phil Singer and Mr. Ned Stone. They are working very diligently to try and get this done in time for EPA to use in its stage two rule making on disinfection by-products. The nice thing about metropolitan Atlanta is the six treatment plants in the five county area have similar sources of raw water; they are all surface water with low bromide concentrations. We know that our primary disinfection by-product for THMs is chloroform. The plants use free chlorine as the primary and secondary disinfectants; they do not have chlorine booster stations in the distribution system, they have low concentrations of inorganic reducing agents, and most of the chlorine demand is exerted by the natural organic material. We're in the second year of this study which is evaluating the link between disinfection by-products and the occurrence of birth defects in metropolitan Atlanta. It's based on historical MACDP data back to 1968 and also historical water treatment data with a validation study. A modeling component by Singer and Stone is evaluating the correlation between historical THM concentrations in chlorine consumption, and using those correlations to predict exposure at the individual level (at the subject's home) for both THMs and HAAs. In the validation study they are actually going out and measuring THMs and HAAs as well as the individual residual chlorine at the tap; it's a very good validation effort. The exposure

assessment is based on this relationship: chlorine consumption equals chlorine dose at the treatment plant minus the chlorine residual at the tap. It's been shown in metropolitan Atlanta and elsewhere that the residual chlorine concentration in the distribution system varies inversely with the THM concentration; we're using this as a basis for our model. The purpose is to review the chlorine dose and chlorine residual records at each utility and use these to provide a very strong framework upon which to build our exposure assessment. Large cities like Atlanta, as most of you know, have very stringent requirements for routine monitoring of their water distribution systems as part of their normal pathogen control program. In metropolitan Atlanta, each of these six distribution systems takes hundreds of samples monthly to look at individual chlorine consumption, chlorine dose and chlorine residual at the tap. In contrast, the numbers are much smaller for THM measurements. A couple of counties do 6-8 measurements monthly, but most counties do 1-2 samples quarterly. You can see that we're going to have a lot more information on chlorine consumption, chlorine residual, and chlorine dose from the treatment records versus the actual levels of THMs in the distribution system or at the water treatment plant. We need to basically build on the strength of this data set. We want to evaluate and refine the relationship between chlorine, THMs and HAAs. I use these correlations to predict exposure at the residence of each of the birth defects cases, and use geographic information systems to map chlorine consumption. We evaluate THM and HAA concentrations on a monthly basis, and do spatial statistics, transurface analysis, and spatial prediction techniques to evaluate the relationship between birth defects and tap water.

To give you an idea of the daunting task we have in front of us, we have ongoing abstraction of 325,000 exposure data points. We have all THM data for the 1990s, most available THM data for the 1980s, some recent chlorine demand data for Cobb County and all the chlorine demand data for Dekalb County. We're going to start with Dekalb County as a pilot and get the kinks worked out of our analysis. Currently the THM levels range from 15-60 to a maximum of 130 micrograms per liter, so there is a decent range of exposure. We've demonstrated the linear relationship between THM formation and chlorine demand that we've seen in previous studies; it's been confirmed but it needs improvement. The modeling work continues and we've initiated our sampling program for specific THMs and HAAs. You can see the relationship - as chlorine demand increases so do Total THMs. We mapped all of the chlorine demand levels in the Atlanta area with their associated THMs and you can see there's generally a linear trend, but there's quite a bit of variability with the specific chlorine demand level and a specific Total THMs. If you wanted to predict the Total THM level a mom is likely to be exposed to at the tap, we can use the particular chlorine demand that is occurring in that area. It would be difficult to pin it down; we need to do some more work.

We are also using the Krieging technique, which is a spatial prediction technique that allows you to take individual data points and interpolate values for areas without data. We don't have a measurement at each mom's residence, so how do we decide what that mom was exposed to? Krieging defines a gradient where the levels of chlorine demand increase as you move in a certain direction; lines can then be drawn of equal predicted levels of exposure. In this way we'll be able to group residences and overlay the information by residence and hopefully improve the prediction of the exposure at that residence.

Let's bring the focus back to a broader level to explain why we're doing this. We feel it's important for EPA to have more data available to them as they move into the stage two rule making process for disinfection by-products. We shouldn't base our policy-making on one epidemiologic study. Basically, that's what we have available to us with respect to birth defects. Our group feels it's really important for us to contribute in any way we can to improving our scientific understanding of this issue. What's needed are additional well designed studies that will assist EPA to propose the most appropriate disinfection by-products and assist them to focus their limited resources most effectively on drinking water contaminants which pose the largest human health risk. Even a relatively small increase in risk of adverse reproductive outcome, if confirmed with additional studies, indicates there's a substantial prevention opportunity when we consider the number of women at reproductive ages in the United States.

Infectious agents in drinking water and control of disinfection by-products

Benito Marinas, Ph.D., University of Illinois at Urbana-Champaign

Dr. Marinas is Associate Professor of Environmental Engineering in the Department of Civil Engineering at the University of Illinois at Urbana-Champaign. He received his B.S. in civil engineering from Madrid, Spain and M.S. and Ph.D. Degrees in environmental engineering from the University of California, Berkeley. He teaches and researches in the areas of water chemistry, physical-chemical treatment processes, heterogeneous photocatalysis, membrane separation and oxidation reduction. He has written numerous peer reviewed articles on disinfection technologies and other drinking water treatment research. Prior to joining the faculty at the University of Illinois, Dr. Marinas was a faculty member at Purdue.

I would like to discuss some of the engineering aspects of disinfection and the control of microbial contaminants and disinfection by-products (DBPs) in drinking water. The control of DBPs and microbial contaminants are related. The EPA, through the Safe Drinking Water Act, addresses this in the Microbial Disinfection By-Products Rule (M/DBP). Essentially, control of both of these is desirable and a balance needs to be struck. I think that is the spirit of what Dr. Cantor indicated in his presentation earlier. There is full agreement that protection is needed against microbial contaminants in water and that disinfectants are needed to achieve this. Engineers have the task of designing processes to control the formation of disinfection by-products. With this in mind, the committee that developed the M/DBP has agreed almost unanimously to address it with a multi-barrier concept. The committee was concerned about *Cryptosporidium* (*crypto*) in particular. However, solutions to that problem and control of DBPs are not available yet.

The multi-barrier concept includes three components. First, protection of source water and watersheds is needed. Second, a reliable method to physically remove microorganisms is necessary. That's roughly a two log removal and a reduction in maximum turbidity from 5 to 1 NTU. Conventional filtration can do that, coupled with coagulation, and sedimentation. The practice of enhanced coagulation helps, too. This is one of the angles that can be brought to the control of microbial contaminants and disinfection. The DBP precursors can be produced by practicing enhanced coagulation. Reducing turbidity and removing more of the particles, including microorganisms, is also important. There are emerging technologies available like membranes, which are able to remove disinfection by-product precursors, and particles including microorganisms. Finally, the third component of the multi-barrier concept is inactivation, either chemical or radiation.

Designing a strategy to control both microbial contaminants and DBPs to this point has failed. For example, disinfection is needed for *crypto*, but it is quite resistant to disinfection. It is not known how resistant it is. In order to look at the global aspect of disinfection and DBP control, a better understanding of disinfection technologies is needed. Essentially, I'm talking about *crypto* and protozoans. In general, it is not known how to distinguish between a protozoan that has been exposed to disinfection and inactivated from one that is not disinfected. There are many challenges in the area of inactivation of *crypto*. *Crypto* have a very complex life cycle. When they are inside the host, a human being or animal, they go through a very complex cycle, but once they get outside into the environment through feces, they become dormant. The *crypto* oocyst is very resistant to toxic environments, to the attack of oxidizing agents. The oocyst is protected and can last a long time. So, disinfecting with chemicals is not the way to tackle this type of microbial contaminant, because they are designed to resist that. However, it is felt ozone could do the job among the various disinfectants, if disinfection is included as part of the overall strategy to control *crypto*. However, some of the chlorine species are not very effective at all. Developing a strategy that will allow traditional chlorination processes by using sequential disinfection is one way to address this problem. Essentially, the oocyst is very resistant but the microorganisms that it encloses are not. If somehow a change in conditions is created to bring the *crypto* out of the dormant stage, chlorine and chloramines may work. So that's one of the challenges.

There are also different *crypto* species. *Parvum* is well defined as a human pathogen, but there are other species of *crypto* that have some doubt still. Some may be much more resistant than *crypto parvum* to disinfection processes based on preliminary findings. There are many other microorganisms that may have to be taken care of and some of them may be even more resistant, so it's a big challenge. For *parvum* itself, the literature continues reporting that different variations could

possibly have different resistance to disinfection. I'm not too sure that is actually the case, I'll address that with an example later on. It may be due to how the oocysts are prepared when laboratory experiments are done. Nevertheless, that is one of the concerns. In addition, use of different methods may produce different results. It is still not understood very well how to assess *crypto* inactivation - the difference between *crypto* that is infective and *crypto* that is not. There are in vitro methods, animal infectivity methods, molecular probes, and different types of dyes. There are strong discrepancies between these methods. The bottom line is that when an overall strategy for disinfection and DBP control is devised, it has to be applicable at the fullest scale. Since observations show that it is quite difficult to meet this standard, a strong safety factor is not something that is called for. A better handle on these processes at the fullest scale is what is needed. Unfortunately, someone will probably go to jail if it is attempted to make the fullest scale with *crypto* in a treatment plant. So addressing the use of surrogate indicators is another way to approach the *crypto* issue. Our group has published a paper on the use of non-biological indicators called microspheres. These microspheres actually simulate the disinfection efficiency of *crypto*.

Let's talk about how much disinfectant is needed to disinfect adequately. First of all there is a lot of variability in what is observed. *Giardia lamblia* is a microorganism that currently is regulated in drinking water and under the surface water treatment rule. *Giardia* is considered to be a challenge. Some utilities have switched to ozone to manage *giardia* but when *crypto* is compared to *giardia* it is much more resistant. It is essentially resistant to chloramine and free chlorine. Chlorine dioxide does a little bit better, ozone is the only one that appears to be more effective. Nevertheless, it is still somewhere in the order of magnitude of 10 times more disinfection required to get at the two log inactivation. So, it is a challenge. Some utilities have switched to ozone to meet the *giardia* challenge. Yet, their design cannot meet *crypto* inactivation. They are going to have to upgrade those ozonation plants to meet the *crypto* challenge. Experiments in our laboratory have been performed for the inactivation of *crypto parvum* with ozone. Two different sources of *crypto* were used. Our experiments ran out of the ones that we were using, so a different source was used and we found somewhat different results. The more resistant curve comes from the EPA. It is a strain that has been used for many years and it has been produced by infecting mice and recuperating it from the feces of mice. The other one came from cows that have been infected in nature. Our results showed there is a factor of nearly three in the inactivation efficiency, so it's homogenous in terms of the requirements for disinfection. The methods used in the process of preparing microorganism from the feces of animals to be used in the study were different. Both methods exposed the microorganisms to a lot of chemicals. Some of those chemicals may have damaged some of the microorganisms. That could be a reason the results from the two groups are different. We still don't have an answer.

How is inactivation at full-scale assessed, and more importantly, how does the control of disinfection by-products fit? Methods from preliminary work have been published in the *Journal of American Water Works Association* on the use of microspheres as non-biological surrogate indicators. This is a schematic of the analogy created. *Crypto* is rarely encountered and difficult to detect in the water going to a treatment plant. It is almost impossible to assess what level of inactivation disinfection agents gave. The disinfectant could be ozone or it could be chlorine. From a mechanistic point of view the disinfectant must first mass transfer to the surface of the microorganisms. The oocyst has a very strong protective layer that the disinfectant will then have to penetrate. It is a very effective barrier, highly protective for the microorganism. But eventually, with sufficient time and with sufficient disinfectant, it will reach inside the delicate microorganism and be effective and the inactivation process will take place. This can be simulated with a much simpler tool. This tool is essentially a microsphere made out of polystyrene. Inside the microsphere there is a fluorescent dye that is very reactive with disinfectants, ozone included. The process for ozone involves it being transferred to the surface of the sphere. It has to then start to penetrate, reacting with the polystyrene matrix. It will also react with the fluorescence resulting in a decrease of fluorescence. We have designed these tools to match the inactivation of the microorganisms. Since it is unknown what that is, there will be some variability depending on the method used or the strain used. There are microspheres that can actually match the kinetics of inactivation of these organisms. So this method is much simpler. This process is not identical to the process going on with the microorganism- but there are some similarities. If one can be calibrated against the other, it could be used as a tool in a full-scale contactor to show the level for *crypto.* At the same time making sure to minimize the formation of disinfection by-products and optimizing the

performance of the disinfection process can be designed into the process.

One concern in full-scale ozone contactors is that there are a lot of non-idealities. What happened in those large units will not be similar to what is observed in a small, well-controlled unit in the laboratory, primarily due to the hydrodynamics. However, if water that has *giardia* oocyst and microspheres entering the contactor, both would undergo exactly the same non-idealities in terms of exposure to disinfectant instead of hydrodynamics. The real process is happening for both of them.

I would like to briefly describe a demonstration of this technology in a full-scale unit. The example is the Alameda County Water District treatment plant in California. The unit tested has a maximum capacity of 14 million gallons per day. Ozone was being applied in the first and second stages. The remaining stages were additional contacts. There are different sampling ports where the ozone concentration is being measured. As you can see in the second vessel where ozone is applied there is decay. Eventually from that point on, the ozone will start to dissipate by reacting with the organic matter. With this information the volume average of the concentration can be measured. That average will multiply with the contact time when we perform a tracer test. This gives a curve coming out through the effluent gate. That's called a T-term for 10% of the mass to reach the effluent. The T-term multiplied by the average ozone concentration will give us the contact time (CT). An experiment was done for *giardia* that could actually achieve a run of 2.2 milligrams per liter multiplied by minutes. The next thing under identical conditions was, instead of using the tracer chemical, the microsphere was used and added to the effluent and the system was allowed to reach its steady state. In that experiment the only thing to do was grab a sample from the effluent and measure the decay in fluorescence. The laboratory correlation had been developed and can be used to give an idea of how much inactivation efficiency is achieved in the contactor.

The dots in this figure are actually the predicted inactivation points for *crypto*. Predicted in the sense that there's actually some intercorrelation between actual experimental data. There are data for 10, 15, 20, and 25 degrees, so it was decided that the full-scale contactor would run on 19.9 degrees. Interpolation between those experimental data is the curve for the inactivation for this specific strength. This could change. For that strength there is a microsphere that under identical conditions will decay in fluorescence. You can see that it is not a perfect match, they are two different systems, but it gives a very nice correlation. A graphical method can be used in this case; this dot is the microsphere curve. It is actually the decay in fluorescence observed with a full-scale contactor. It was measured and plotted. Notice that it is slightly above a CT of two, so independently you see microsphere florescent decay. The same CT has been obtained by taking all of those samples in the ozone contactor by running a tracer test and calculating the T-term. So, this gives a way to also confirm or calculate our CT approach with a single sample measuring florescence which is something quite easy to measure. One more powerful thing that this is doing. If it is agreed that this will end up being similar to the inactivation kinetics of *crypto* using ozone disinfectant, then the graphical correlation to estimate the inactivation of *crypto* if *crypto* was going into that ozone contactor can be applied. Essentially, there is an excess of two logs. What this tool is doing is that since *crypto* cannot be added, and at full-scale it is difficult to demonstrate and compare DBP control and disinfection, this tool allows the measurement of DBP under different conditions. It will also compare DBP and disinfection efficiency in the full-scale. That is the next phase of the project. At this point a technology has been developed showing it works, indicating the level of disinfection.

In order to have a comprehensive strategy for both microbial and DBP to implement a multi-barrier concept for *crypto* including inactivation, as well as other microbial microorganisms, a better understanding of disinfection technology is needed, as well as what efficiencies are being achieved. The other point is that indicators can be used as non-biological surrogates as an alternative to CT. The full-scale ozone contactor can be characterized and the technology for all the disinfectants is being developed. Again, it's simple and it gives an accurate indication of what is happening without having to take too much of a safety factor because it takes into account the non-idealities of the system. Finally, the ultimate goal for the future would be to use this tool to represent disinfection. The disinfection by-products can be measured and the process actually optimized with respect to these two concepts.

Industry perspective on disinfection of drinking water supplies

Ed Moreno, City of Iowa City

Ed Moreno is Water Superintendent for Iowa City and has been with the Iowa City Water Division for 7 years. Previously, he worked as a treatment plant operator at the University of Iowa Water Plant and was an environmental specialist and field office supervisor for the Iowa Department of Natural Resources. He holds a master's degree in civil and environmental engineering from the University of Iowa.

(Editor's note: This talk was first presented under the title "Innovative Water Treatment: Today" at the Safe Drinking Water - Iowa's Future Conference on November 4, 1994, at Kirkwood Community College in Cedar Rapids, Iowa.)

Iowa City has a $50 million dollar renovation project to construct a new water treatment plant and do major upgrades on our distribution system. In doing so we plan to accomplish the transformation of water quality in Iowa City. Through our investigations, we've encountered many water quality issues that are common to Iowa. I will touch on those in addition to some other issues that are occurring around the state.

It all starts with our primary source water, the Iowa River. The issues that we face--the taste and odor problems, the contamination --are mostly related to the Iowa River. The Coralville Lake dam is operated by the Corps of Engineers and their mission is related to flood control, flow augmentation, recreation, and wildlife--somewhere in there is water quality. They change the flow coming down the Iowa River 3-4 miles upstream from the intake of the Iowa City Water Plant. These changes have a great impact on what happens at our plant. The Corps saved us during the 1993 flood. We were the focus for their operation during 1993 and they were able to make sure we did not go under like the Des Moines Water Treatment Plant. The emergency spillway is vintage 1974. We see changes in flows coming down the Iowa River ranging from a low of 55 cu.ft. per second to a high during the flood of 26,500 cu.ft. per second. With that comes drastic changes in water quality. Samples taken following a heavy precipitation event in our watershed area show the turbidity we sometimes have to deal with. We've seen turbidity range from a low of 2-3 in the winter to a high of 10,000 after a precipitation event. What you can't see is that in one of the vials is also 210 parts per billion of atrazine and elevated levels of nitrate. We have quite a bit of contaminants at certain times in the Iowa River.

The Iowa City water plant is right in the heart of the University of Iowa campus. The water plant has always been located at this location, in the heart of campus since 1882. It was privately owned until 1961 at which time the city purchased it and embarked on a very aggressive construction project to upgrade the plant. The Iowa City water plant is designed to remove dirt, bacteria, viruses, protozoans and protozoan cysts from the water. Our current treatment process it is not designed for anything more advanced than lime softening. We have a computer control system that allows us to automatically control what is going on in the plant. In addition, we have manual controls out in the plant in case we have problems with our computer system. We have filters that are vintage 1909 and they're still in operation--processing approximately 1/4-1/3 of the water produced daily by Iowa City. The rest is produced out of the new part of the plant. Part of our treatment process allows us to bring deep well water from the Jordan aquifer into the end of the water treatment plant--we utilize it sporadically; sometimes for demand purposes, more often for contaminant purposes. If there are high levels of contaminants in the river, such as nitrate or pesticides or other organics, we will turn on this deep well and bring in Jordan water, which is really high in solids, but has none of the other contaminants.

Currently, we have three modes of operation: totally automatic; where the computer system is programmed to run everything; semi-remote, or remote control, where the operator can push a button in the control room, and something will happen out in the plant; and manual. The plant has a very basic wet chemistry lab where we do our QA/QC and derive samples, which are done on a minimum of four hour increments. We measure where we are and make adjustments from those measurements--we check for chlorine, turbidity, nitrates, pH's, and total dissolved solids. Currently, we are allowed to discharge solids directly into the Iowa River; we discharge thousands of pounds of solids each day back into the Iowa River. Two percent of the water treatment plants in Iowa are at the complexity or the rate we are--grade IV surface water treatment plant.

I'm going to talk about some of the issues that brought us to our decision to proceed with the planning for a new water treatment plant. We normally experience a couple fish kills each year

where there are thousands of fish coming down the river. Back in 1988 and 1989 there was a major drought during which time the water quality of the river was not too bad. There was a lot of algae and stagnation, but contaminants like herbicides, pesticides, and nitrate were very low. However, when it did start to rain we started to see things like foam on the river. A lot of organics came down with source precipitation at that time. We were using chlorine at levels upwards of 1,200 pounds a day, which comes to about 25 parts per million. Ordinarily we see something less then eight. It was a very difficult time for us. The precipitation event caused an influx of nitrate larger than we had ever seen, something in the order of 65 parts per million as nitrate. For us, the solution to pollution is dilution with the deep wells, but we were unable to meet the demands needed to keep nitrate low enough.

Another key factor was the Safe Drinking Water Act amendment of 1986, which indicated that the game was going to be drastically and very quickly changing. It became apparent to us that we needed to do some major planning for Iowa City. In 1990 we decided to create what we call the Comprehensive Water Facility Plant. Our first step was to interview consultants and choose one who we felt had the flexibility and base knowledge to help us create a plan that allowed us to look at all of the issues in place as well as those we knew were going to be coming. Another thing we did was to form what we called a "technical advisory group." This group consisted of individuals throughout Iowa City: professional people, academicians, soil conservationists, the Culligan Man, local plumbers, and industrial representatives. We asked them to give us some direction. These were issues ranging from water conservation, water use rates and regional water issues, to water quality standards. The Advisory Group discussed and prioritized these issues. The basis of our charge is the following: 1) Iowa City drinking water should meet all of the existing and predicted water quality standards; 2) The water treatment plant should produce water that is aesthetically pleasing and environmentally acceptable to the general consumer; and 3) water officials should project what we needed to do improvement-wise 20 to 40 years into the future. Built into these 3 items were water conservation, regional water and other issues. We began evaluating the existing water treatment plant to see what was possible, and started to look at water resources in and around Iowa City. We purchased a site that is approximately 230 acres of land. On that site we'll have our sources and build a new water treatment plant. We are fortunate to have some alluvial sand on the site which would be our primary source. Our intention is to get away from the river as much as possible and utilize sources that are much better.

Our site is on the corner of Interstate 80 and North Dubuque Street. On this site is a quarry where they are mining the sand--they have an alluvial aquifer. We will no longer be allowed to discharge solids into the Iowa River, we will have sludge lagoons. We have done the preliminary investigations to allow us to have horizontal collector buoys. We will be obtaining alluvial water from this site. We're going to be putting a horizontal collector well in a couple of areas on the site, and we're going to install two Silurian pumps. In addition, there is another area we call the peninsula where there is a good alluvial fan that we intend to put two horizonal collector wells in, in addition to two more Silurian wells. We will bring that water into a water treatment plant that will perform lime softening followed by activated carbon filtration. Initially we'll probably go out with prechlorination. With every surface water plant that is designed there's always the future ozone section. Our plant is designed with the future ozone area so that if we see the need in the future to switch to ozonation, the hydraulics will already be accounted for, and we'll just have to bring in the equipment. We'll have a pilot plant in the new plant to continue our studies of issues such as that. We're intending to construct three major feeder lines that will distribute the water differently throughout the city. We propose changing the existing water treatment plant to a water pumping station.

One of bigger issues is related to well head protection--water rights. Who owns the water? Who can get the water? How do you protect people from being impacted from well interference by an entity as large as Iowa City, which is looking for water in aquifers that are already heavily used? Another issue is related to public involvement and public notification. We hope to incorporate valuable information coming from those issues into our future plans. We are in a prime position to make changes. With respect to the well protection and well interference issues we've put together a team of representatives from the USGS, the Iowa Geological Survey Bureau, the Johnson County Health Department, as well as our consultant. Issues related to *Cryptosporidium* and disinfection byproducts problems bring up the issue of balancing acute and chronic risks. In the water industry we're aware of the balancing act that we must do. We're struggling quite a bit with our ability to communicate that. Another issue is watershed management. We have a limited

ability to control and manage the entire watershed and the many accesses to it. There are a lot of activities going on in Johnson County to assist with that and we will be participating.

We will also participate with the watershed and management team and their ideas. We will be proceeding with our water treatment plan. We are going to be on different schedules, but I think we all have a similar mission.

Some other things that are going on around the state: Des Moines has constructed a nitrate removal facility. It's unique, they say it's the largest in the world, and that's one way to address that contaminant. We're looking at diluting it. Also, we've embarked on a watershed management project, where we're cooperating with groups in Minnesota and in Iowa to protect the Raccoon River watershed.

Keokuk is looking Aquifer Storage Recovery (ASR), which involves looking at taking treated water and injecting it into aquifer in the ground to store it and bring it back later. You can run your plant in a certain capacity and then bring it back without having to expand later. Other places are working with the poplar trees and their ability to absorb contaminants that are running off into the streams. Rural water is a very interesting issue, a primary issue is Iowa almost lost the ability to regulate in the state. Just the ability to control our destiny through in-state people is very important. The issue that CHEEC is working on--contaminants and what are their impact is on health I think a real key issue is looking at staffing for the future--the staff that we're going to need to fulfill these kind of plans. We better have somebody there to run things, and they better be on top of it.

Featured Speaker

U.S. Geological Survey research on pesticides in the Midwest

Donald Goolsby, U.S. Geological Survey Bureau

Donald Goolsby serves as Chief of the U.S. Geological Survey's Mid-Continent Regional Herbicide Project, out of the Denver USGS office. He graduated from Florida State University, with studies in chemistry and oceanography. He has worked on numerous hydrologic and water quality studies across the country, from Florida to Virginia and throughout the entire Mississippi River basin. In recent years, Mr. Goolsby has been responsible for the planning and direction of research in the occurrence, transport and fate of pesticides and nutrients in surface and groundwater throughout the mid-continent United States.

I want to thank George Hallberg and the University of Iowa for inviting me here to participate in this really fine conference. The topic that I've been asked to talk about is USGS research on pesticides in the mid-continent. I'll mainly be focusing on pesticides in the hydrologic cycle of the Mississippi River basin, which has very intensive agriculture. In fact, much of its land surface area, in excess of 80% in many counties, is that of harvested crops. Associated with all this intense agriculture is heavy use of agricultural chemicals particularly pesticides and more specifically, herbicides. The herbicide use is much higher on these crops than insecticide use. Five of the most heavily used herbicides in the country are atrazine, alachlor, metolachlor, cyanazine and acetachlor. The combined use of all of these herbicides is several hundred million pounds per year throughout the Mississippi River basin. There has been a downward trend in recent years. Since about 1990, the use of some of these compounds such as atrazine and metolachlor have leveled off. The use of alachlor has gone way down, but it's being replaced by acetachlor, which has similar properties. Over the last six or seven years, state Geological Surveys have conducted a number of regional scale studies on the occurrence, distribution and transport of pesticides, primarily herbicides, in the Mississippi River basin. In surface water these studies have focused on small streams; we've looked at reservoirs and large rivers such as the Mississippi River. We've conducted studies of agricultural chemicals in groundwater across the Midwest and we've looked at rain water. More recently, we've studied volatilized pesticides in the air in the Mississippi valley. I'll be talking about some results from these studies.

I'd like to begin my talk with a brief description of the process. We start with application of pesticides to the crop or to the land surface, and then a number of things happen from that point. First of all, microbial and chemical degradation of pesticides begins to occur in the soil zone shortly after application. With rainfall, some pesticides and their

degradation products runs off into streams. Some leaches into the groundwater system, and many pesticides volatilize into the atmosphere. In the atmosphere some - depending upon their properties - will attach to soil particles and are transported great distances, hundreds of miles from their point of origin. When it rains, these can be deposited in lakes and on ground surfaces at a great distance. The process that occurs shortly after field applications, and is most noticeable, is that rainfall will often flush large amounts of herbicides into streams. I'm going to use atrazine as an example because it's the most heavily used pesticide in the Midwest and it's also the most persistent pesticide. Atrazine degrades more slowly than most of the other compounds that we see, so it's a good indicator of what's happening to pesticides. Shortly after application, when we get a spring rainfall, a lot of the atrazine and other herbicides are flushed into streams. We may find very high concentrations - 10 to 50 parts per billion - for brief periods of time. These tend to be short pulses that quickly decline when the spring flow goes down; a subsequent storm event can cause another pulse. This process happens for several months following the application of pesticides. The compound dissipates, there's degradation, and uptake by the plants. Concentrations of chemicals like atrazine never really go to zero, we can detect them at some level year round, but very low levels. Later in the year, with rains during November, December and January, there may be no movement of atrazine into the stream. It's all been dissipated by various mechanisms. In April or May of the next year, this process starts over again. If you look at almost any stream across the Midwest, you'll see this same pattern occurring.

The USGS has sampled fifty basins a number of times across the Midwest. They were selected statistically to represent a random sample of streams across the region. The concentrations here represent the post-planting flush; these tend to be near maximum concentrations. This is greater than 12 micrograms per liter, or 12 parts per billion, which is four times the drinking water standard for atrazine. These high concentrations occur all across the Midwest, from Nebraska, Iowa, Illinois, Indiana into Ohio. This sampling was done in 1989 and has been repeated a number of years. The latest year was 1995 and the same pattern persists; there's been very little change across the Midwest. As you go further north, where the intensity of atrazine use decreases, these peak concentrations decrease. Atrazine is just one of the compounds we find. It has the highest median concentration, at about 5 parts per billion. The 75th percentile is up around 11 or 12 parts per billion, and we have some concentrations that extend up to 50 or 60 parts per billion. Other compounds, in order of concentration after atrazine, include a degradate of alachlor (alachlor ethane sulphonic acid) with a median of about 2 parts per billion, followed by metolachlor, cyanazine, two atrazine degradates; deethylatrazine and desisopropyl atrazine, which are both chlorinated compounds. Then there's a cyanazine metabolite (cyanazine amide), acetachlor, alachlor, simazine and finally metribuzin. This is the occurrence pattern of compounds by concentration in these small streams in the Midwest, and this occurs year after year. If you go out in the springtime, just before the planting, you're still going to see some of these compounds in streams. The alachlor metabolite seems to be the most persistent of all of the compounds that we've looked at in this analysis with a median concentration up around 1 part per billion, which is a residual from the previous year. Metolachlor, cyanazine and alachlor are much less stable than atrazine even though they are applied in similar quantities, they degrade much faster; but we found their metabolites in the environment. The runoff from these streams, in many cases, discharges into reservoirs. There are hundreds, maybe thousands, of reservoirs across the Midwest that store water for flood control or for water supplies. The Iowa City water supply depends on one of these reservoirs.

These data are from the drainage basins of about 76 reservoirs that we studied in 1992-93. Reservoirs store and control the flow of water; they also store and control the flow of pesticides. For a small reservoir on a large stream, the concentration of pesticides will look something like the stream. The peak concentration in a reservoir won't be as high as the peak on an unregulated stream, but the base level will be elevated a little bit for a longer period of time. The larger that reservoir is relative to the size of the stream, the longer the pesticide will get stored in the reservoir. This is a reservoir in Illinois that has an average residence time of about 2 months. The sampling period started in April, 1992 and went through September, 1993. You can see that we have high concentrations; the spring flush that came into that reservoir elevated the concentrations to about 6 micrograms per liter. It took nearly a year, at this particular time, for this atrazine to flush out of the reservoir. In April, 1993, we get the spring flush again and the concentration goes back up. Cyanazine behaves somewhat the same way but there's much less cyanazine present in this watershed relative to atrazine. Let's look at the other extreme. This is a

reservoir in Nebraska that has an average residence time of 18 months. If you look at atrazine, there's very little variation and very little flushing of the atrazine out of this reservoir; the concentration ranges from about 2-3 micrograms per liter over the year and a half period. So, reservoirs that get very little flushing can collect the spring pulse of pesticides, and depending on how fast they degrade, can store these pesticides for long periods of time.

Compounds like atrazine, once they are in the soil zone, degrade fairly rapidly. I think atrazine has a reported half life of about 60 days in the soil, but in the water column that half life is very long - it's years. Some recent work on the Great Lakes has shown that the atrazine level in Lake Superior is on the order of 3-4 parts per trillion. The only input is atmospheric, and the lake has a residence time of over a hundred years, so the concentrations are slowly rising. They're not being flushed out, they are slowly building up. The estimated half life of atrazine in Lake Superior is on the order of decade or more. About 10% of the reservoirs we studied have an average annual atrazine concentration greater than 3 parts per billion; about 15% have average atrazine concentrations between 2-3 parts per billion. A lot of reservoirs across the Midwest have atrazine concentrations which exceed the MCL of 3 micrograms per liter on an annual average. Some of these reservoirs are used for public drinking water supplies. These reservoirs can slowly release water with high concentrations of chemicals all year long, so that downstream water intake can certainly be impacted. For example, runoff from these small streams is collected in the reservoirs and discharged into large rivers such as the Mississippi, the Ohio, and the Missouri. In 1991 and 92 we analyzed for about 40 pesticides in the Mississippi, Ohio and the Missouri, and 30 pesticides were detected. We used a detection level of two parts per trillion. The compounds that show up are very similar to what we saw in the smaller streams. There were several herbicides for which 25% or more of the samples had concentrations above the level of detection, including atrazine, alachlor, cyanazine, metolachlor and simazine. We also see a number of insecticides in the Mississippi River, such as carbaryl, carbofuran, parathion, and malathion. We see quite a bit of diazanon, particularly in rivers like the Illinois River and the Ohio River; it used to be associated with urban use. So there are a lot of compounds. The good thing is that most are at low levels; the average annual concentrations in the large rivers do not exceed any of the drinking water standards.

Let's look at concentrations in the Mississippi River at Baton Rouge, which are the levels of these compounds being discharged to the Gulf of Mexico. Concentrations of atrazine and cyanazine range from 1 to 5 micrograms per liter. The annual cycle is apparent here; every year there is a peak and over time it goes down to very low levels, but not to zero. The magnitude of the peak is related to climatic conditions; the amount of rainfall that occurs in a particular year determines the peak concentrations, not only in small streams but also at the mouth of the Mississippi River, which is integrating the entire basin. 1991 and 1993 were wet years. We had peak concentrations for a few weeks, exceeding 3 micrograms per liter. 1992 was a fairly dry year, and the concentrations only got up to about 1.5 micrograms per liter. This is an annual cycle of the compounds being flushed to the Gulf of Mexico. Knowing the concentrations and the flow of these compounds, we can estimate the annual mass flux of these compounds into the Gulf of Mexico. I'm going to focus on atrazine. The annual flux of atrazine ranges from a little over 200 metric tons per year in 1992, the dry year, to about 900 metric tons in 1993, which is the year of the big flood. What percent does this represent of the amount of atrazine applied in the Mississippi basin each year? It ranges from about 1 percent in a dry year like 1992, to a little over 4 percent in a real wet year like 1993. The average is slightly less than 3 percent, so about 3 percent of the atrazine that's applied ends up in the Gulf of Mexico. For the other compounds that percentage is smaller: for cyanazine, the maximum is about 2 percent, for metolachlor it's about 1 percent and for alachlor it's just a fraction of 1 percent. Alachlor is the least stable of these compounds; very little alachlor makes it to the Gulf of Mexico.

Now let's take a look now at another part of the hydrologic cycle - groundwater. In 1991 and 1992, my USGS colleagues here in Iowa and elsewhere conducted reconnaissance of pesticides in groundwater. Covering parts of a twelve state area, 300 wells were selected in a well designed statistical study. About 25% of these wells had detectable levels of atrazine; the good news is that none exceeded the drinking water standard of 3 micrograms per liter. The concentrations were generally quite low; less than 1 microgram per liter. The number of detections depended on the analytical detection limit being used. This study had an analytical detection limit of .05 micrograms per liter, which gave us percent detection of about 25%. Using methods that have lower detection limits, we have found atrazine in about 40-45% of the samples for the same series of wells. So if you lower your

detection limits you're going to find more of the compounds. We're also finding that there are a lot of degradates in groundwater. In fact, the problem in groundwater is mainly degradation products and not parent compounds. Data from 100 of those 300 wells that were sampled in 1992 demonstrate this. Of the six most frequently detected compounds, five were degradates, including alachlor ethane sulphonic acid, two atrazine degradates, an alachlor degradation product, and the degradate of dacthal. Then we start to get into some of the parent compounds. Very few insecticides were detected. In 1995, Dana Kolpin from the USGS in Iowa did some work with George Hallberg at the University Hygienic Laboratory, and the IDNR-Geological Survey Bureau. Within the Iowa municipal well monitoring network, 106 wells were sampled in the summer of 1995. Seventy percent of those wells had detections of some pesticide compound, most of those were degradates. A sub-set of those wells was sampled again in 1996. The two most frequently detected compounds were metolachlor and alachlor, then ethane sulphonic acid, metolachlor and alachlor oxynilic acid, atrazine, deethylatrazine, a cyanazine degradate, an acetachlor degradate, two more atrazine degradates, and finally, metolachlor and prometon. There's a lot of degradation going on in the soil zone and very little of the parent compound, except for atrazine, makes it to the groundwater. The good thing about many of these degradates is they are dechlorinated - the degradation process removes the chlorine. In the case of the ethane sulphonic acid, it's replaced with a sulphonic acid group; the oxynilic acid also results in the removal of the chlorine. In the 1996 study, the highest concentrations were for alachlor ethane sulphonic acid and metolachlor ethane sulphonic acid. A few wells had very high concentrations of these degradates; in the range of 5 to 10 to 20 micrograms per liter. There were also some very high concentrations of the metolachlor oxynilic acid in these wells.

I'm going to move on to the final part of the hydrologic cycle, which is the atmosphere. In 1990 and 1991 we conducted a study of herbicides in rain. We collaborated with the National Atmospheric Deposition Program National Trends Network, and we obtained weekly samples of precipitation from 86 sites over a period of about 19 months. The temporal patterns of herbicides in rainfall are almost identical to what we see in streams. This is a rainfall site in Illinois. Alachlor concentrations in 1990 go up to 2-3 micrograms per liter for a period of 2-3 months, then back down to almost non-detectable levels. Using this analytical method, they were not detectable after late July. The following year the pattern is repeated. While the herbicides are on the moist fields, there's volatilization into the atmosphere. Some compounds, like atrazine, attach to soil particles. Rainfall can flush them from the atmosphere back to the land surface. A number of compounds were detected: atrazine was detected in about 30% of the samples, alachlor in 20%, deethylatrazine in about 20%, metolachlor in about 15% and cyanazine in about 8%. The concentrations tend to be low. Only about 1% of the sample concentrations exceeded 1 microgram per liter. We used a spatial pattern of the precipitation weighted concentration, which is equivalent to collecting all of the rainfall that fell during May and June in a large container and then taking a sub-sample out of it, so it gets weighted by the amount of rainfall. The reason for doing that is the first few millimeters of rainfall scavenges the atmosphere, flushing the pesticides out. If you were to throw that away and collect the later rainfall, you wouldn't find anything. The first rainfall flushes it out and the later rainfall tends to dilute the concentrations in the earlier rainfall. Weighting it by the amount of precipitation you can get something that you can compare. In May-June 1990 and May-June 1991 we see similar patterns. The weighted concentrations are more than 0.3 parts per billion. Some concentrations get up to 1 or 2 parts per billion in the rainfall, but generally they are much lower. The patterns are fairly similar from year to year. In the 1990-91 study, in which we looked only at rain, the detection levels were fairly high, about .05 micrograms per liter or 50 parts per trillion.

In 1995 we conducted another study in which we sampled rainfall and air. In the air, we separated the particles from the vapors so we had air particles, air vapors and rainfall. We had two sites in Mississippi, one in the city of Jackson and one out at the Delta, where a lot of cotton is grown. We had two sites in Iowa; one on a building in Iowa City, and one at the Cedar Rapids Airport. We had a site in downtown Minneapolis, one at an ag site near Princeton, and one on the south shore of Eagle Harbor. That was our way down wind site. We analyzed for 49 pesticides in the air. We had detection levels down on the order of a tenth of a nanogram per cubic meter; that's a very, very low level. We detected 37 pesticides, including the common ones like alachlor and atrazine. We also found a lot of insecticides; carbaryl, carbofuran, chlorpyrifos (which is used for termites,) lindane, DDE, a degradate of DDT, which is no longer being applied but is still volatilizing from the soil and appearing in air samples that we collect.

One of the most interesting sites, because of the number of compounds detected and the concentrations, was down in the Mississippi Delta area. This study was conducted between April and September 1995. The air samples and rainfall samples were composited over a period of one week, so the concentrations represent average weekly concentrations in the rain and average weekly concentrations in the air. We found an average concentration of methyl parathion on the order of 5-10 nanograms per cubic meter for a period of a couple of months. We don't know the environmental health significance of being exposed to these levels of insecticides and herbicides in the air. You're breathing them in and there is nothing to compare against like there is for drinking water - we have drinking water standards. We found other compounds, including propanol, a rice herbicide, malathion, another organophosphorus insecticide, and metolachlor. Finally, I want to point out that DDE was detected in almost every sample at low levels, less than 1 nanogram per cubic meter. It's volatilizing from the soil. A lot of DDT was used in the cotton area in the past, it's not used any more, but it's slowly volatilizing out of the soil. Almost every air sample that we collected had this level of 4,4'-DDE. I wanted to contrast the urban site in Jackson with the agricultural site out at the Mississippi Delta. Every single urban sample had fairly high levels of chlorpyrifos, which is used for termite control in houses. A few of the sites out at the Delta had chlorpyrifos, but nothing like in the urban area.

Finally, I wanted to say a little bit about Iowa City. In Iowa City and at Cedar Rapids, the compound we detected at the highest concentrations in air and in rain was acetachlor. Concentrations weren't high; in rain there were about 1.5 micrograms per liter for the highest concentrations; in air we found concentrations of 2 - 4 micrograms per liter. Acetachlor is a relatively new herbicide, introduced in the last three or four years, and has the highest concentrations in air throughout this area. I want to summarize the data from Iowa City. In the 19 air samples that were collected over the six month period, we detected 23 pesticides in the air and 25 pesticides in the rain. Four compounds were detected in every single one of the Iowa City air samples: trifluralin, benfluralin, metolachlor, and DDE. The pattern was a little different out at Cedar Rapids but I won't go into that. In 80% of the samples we also detected dieldrin, which is another old chlorinated compound, and over 80% of the samples had chlorpyrifos.

To summarize, what I've presented is the current distribution of pesticides in the hydrologic cycle. They occur in all compartments of the cycle, in rain, in the air, in surface water and in groundwater. In groundwater the concentrations tend to be relatively low; they do not exceed - in most of the work we've done - drinking water standards. Drinking water standards are exceeded in small streams and in reservoirs but generally not in the large rivers. We also find a lot of pesticides in the air. At this point we don't know the significance of some of the concentrations we're seeing in the air. We haven't published the air data yet, most all of the other work has been published. We'll be coming out with something on the air study in the near future.

Session 2: Synthetic Organic Chemicals and Birth Defects

Toxicant-induced birth defects: What animal models can teach us

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(Editor's Note: Dr. Sulik's presentation relied upon the use of slides. Much of the richness and texture of her presentation may be lost in this transcription. Readers are encouraged to access refereed journal articles by Dr. Sulik.)

The title that I've selected for my talk today, I'm afraid, is somewhat misleading, because it perhaps indicates that I'm going to tell you something about chlorination by-products and exactly what those can do to embryos. Unfortunately, I'm not going to be able to tell you about a particular compound causing a particular birth defect in a particular way, at least the compounds that all of you are most concerned with. Instead, what I'd like to do is provide for you a framework through which you can begin to understand the genesis of particular kinds of birth defects, like cleft palate, neural tube defects and cardiac malformations. To understand a little bit of the embryology, to think about gene environment interactions, to begin to appreciate the complexities of development, and also to appreciate the state of the art of developmental biology, and the opportunities that it offers to us. To begin to understand what particular compounds do to the embryo at a particular dosage level, at a particular time in development.

To put that into a little bit of a framework, I want to quote a few statistics, although the epidemiologists here may argue with the numbers. We usually say that about one in thirty-three babies is born with a serious structural abnormality. We also say that the leading cause of infant death in this country is birth defects, because we've conquered a lot of the other problems like infectious diseases. In spite of recognizing that birth defects are the leading cause of infant mortality, we still don't know what causes the majority of our birth defects. Certainly, we can say that many of them are caused almost purely by genetic abnormalities, some are caused almost purely by environmental insults, some are caused by an obvious combination or a gene-environment interaction, and for others, we really don't know. We do know that abnormal development can result from an insult at all the different stages of embryogenesis. This is in contrast to a previous belief that has only changed very recently, that at pre-implantation stages (from fertilization until the second week of human development) the embryo will be affected by an environmental insult or even a genetic insult by either dying or being completely normal. We refer to it as an "all or none" kind of phenomenon. Now, as a result of a number of studies in teratology laboratories, we know that is not the case. The embryo is, in fact, vulnerable to insults that can cause major malformations through which it can survive and be born at these early stages. It's during the embryonic period that we cause most of the major malformations and major structural abnormalities that we see as obvious birth defects. The embryonic period in humans extends from about the third through the eighth week of development. We're aware that at fetal and even at postnatal stages we can cause abnormal development; we usually categorize these as being disruptions or functional deficits. However, we have to recognize that we can cause functional deficits at the earlier stages as well.

What I will do is start at the stage of development when we already have some of our cellular building blocks. Taking all of those little particles as they rapidly divide and build is something that we can readily identify and recognize as a human form. So we are going to have a bit of an embryology lesson, and then we're going to see how some things can run amok. We're going to start with a few diagrams and then we'll progress to some scanning electron micrographs.

This slide represents a human embryo; most mammalian species develop very, very similarly. The embryo is implanted in the uterine wall. The part that

actually is the embryo that will become the individual consists of three layers of cells - the upper layer, the middle layer, and the lower layer. The amniotic cavity, through which we do amniocenteses, is above the upper layer and the yolk sac cavity, which is associated with the gut, is associated with the lower layer. When we're at that flat layer stage, before we have that middle layer, there are two layers; the upper layer and the lower layer. The upper layer is in contact with the amniotic cavity, the lower layer with the yolk sac cavity. If we look at that whole individual, it's like a bi-layered disc and in that bi-layered disc, those cells already know what they're going to become. They're programmed, although under the influence of other cells at later stages they can change their commitments or become differentiated. Very early on, one end of the disc is where the heart will form. The heart is the leading structure in the embryo, and behind that is the region where the mouth will form. The heart leads the mouth at this early stage. In the midline at the back end of the embryo is a very special region called the primitive streak.

It's from that primitive streak that cells from the upper layer ingress to make the middle layer of cells. If we look at a real embryo at this stage, we can see the two layers of cells. The individual granularity you see here are individual cells of this embryo. The rounded area is called the pre-quartal plate. That is where the oral cavity region will be. The heart will be forming way out here. There is a line of distinction all along here; these cells look a little different. This is the line that defines the surface ectoderm (that tissue that will cover the outside of your body) from the neural ectoderm (that tissue that will be inside your brain). We're essentially two tubes; we start out as a flat plate made of two layers. The upper layer has to round up and make a tube; that's your neural tube, your brain. The lower layer has to round in the opposite direction and make a gut tube. Everything else that surrounds it is merely filling. This is the line along this edge that will have to come to a corresponding line along this edge to round up and make a neural tube. If we make a cut through the tail end of the embryo we can see the third layer of the embryo. Each layer looks a little different. The upper layer is called the ectoderm, the middle layer is the mesoderm, and the lower layer is the endoderm.

As the embryo gets a little longer (it's still quite cup-shaped) the part that is going to make the neural tube, the neural plate, seems to be growing. The line of distinction between the neural and surface ectoderm is right along here. It will eventually come together and unite to make a tube. The embryo gets longer in a head to tail direction, because more and more cells are added from the primitive streak. It's sort of like squeezing ourselves out of a toothpaste tube; we get longer at the tail end and our head end develops faster compared to the tail end. If we look at this embryo and we make a line right there, this is all brain. We haven't made much of the caudal end of the embryo yet. The brain includes the forebrain (which is going to be the whole cerebral hemisphere), the midbrain and the hindbrain. If you tip the embryo a little bit to the side, you see that as the embryo elongates from front to back, the brain is growing much more rapidly than the heart and the mouth. The brain grows real fast and as a result the heart and the mouth get carried sort of down and forward. The two cerebral hemispheres will need to unite. Here is the line between the neural and surface ectoderm; if this is the brain, then this must be the outside of the face. Here are the three germ layers, the ectoderm, mesoderm and endoderm. This is starting to form what will end up looking like a tube. A little bit later in development, this part of the forebrain is growing very rapidly. There are two little indentations there that represent where the eyes are going to form, because eyes are really an outpocketing of the brain. Here's where the oral cavity (the mouth) will be, and here's the heart. This embryo corresponds to a human at the beginning of the fourth week of development, and at that stage the heart is starting to beat.

The next slide shows you what size that embryo is. This is an embryo mounted next to a penny. This is a mouse embryo but a human really wouldn't be much different in size at this stage in development. At four weeks when your heart is beating, when your mother probably doesn't know yet that she's pregnant, you're about the size of the number one on the penny. But you've established all the tissues that are going to make your brain, your heart, and your gut, and your form is becoming very defined. At that stage, if we look at the embryo from the dorsal side, none of the neural tube has closed yet to make a tube. Instead, it's really much like a plate or a neural fold. The folds are already fairly close together, and it isn't going to take much to get them close enough to fuse. As the neural tube closes, starting in the occipital region, it begins to zip up both forward and backward. The embryo is getting a lot longer at this stage. If a cut is made through the head end of the embryo right to the point where it was just getting close enough to start to fuse forward, we can see the layers of cells. The cells that populate each layer are beginning to become very distinctive. Here is the surface ectoderm, and that's the neural ectoderm. The surface ectoderm becomes very thin; the neural

ectoderm stays very tall columnar cells. At the top of the neural fold is a special population of cells called neural crest cells. These cells, which were originally part of this very organized epithelium, start to leave the epithelium and come into the middle space to make another very important population. If the neural crest cells were not made, you would not have a face, because the crest cells make all the skeletal and connective tissue in your face, with the exception of the tooth enamel.

This is a neural tube that has closed. Right at the top of the tube, we can see that cells look like they're melting off the epithelium, becoming mesenchymal or fibroblastic, and moving into this middle space. In the head region, even before the neural tube closes, the neural crest cells begin to migrate into the middle space underneath the surface ectoderm and populate the parts of the head that will form the upper and the lower jaw and the front of the face. Some of those neural crest cells will migrate into the developing heart. If there is a problem with neural crest cell development or migration, we can end up with heart defects as well as craniofacial defects. The embryo begins to curve in the direction we think of as the normal fetal position, with knees up by the nose. The neural tube is beginning to close as the two forebrain hemispheres start to come together like a clam shell. Here is the forebrain area that covers the area that will be the nose, the mouth is in here, and here's the heart and it's beating. We look at an embryo at this stage from the gut side and appreciate that the edges of those folds are coming very close together, and they'll need to unite in the midline. The time that they do unite in the midline is right around twenty-five days in a human; the last place to close is called the anterior neuropore. At this stage, your nostrils should be at almost the far lateral aspect of your head. If they're too close in the midline, that indicates that you've lost a lot of the midline tissue of your face and your brain. These dents that are seen at a later stage are going to be the inside of the nostrils. The tissue that will make the tip and side of your nose will be built from this area. This will be your upper jaw and your lower jaw, and this will be your pituitary gland. In a later stage, it looks a little more like a nose with nostrils, but you see that they're widely spaced. At this stage in development, there is a big ditch in the midline between your nostrils. Here is the pituitary gland, the upper jaw, and the lower jaw. Later on, the nose seems to be getting pudgier, but in fact we still have this big groove in between the nostrils, which is normal. In order to form a normal upper lip, one without a cleft, the tissue that was on this side of the nostril (the medial nasal prominence) has to be brought together with the tissue that was on this side of the nostril (the lateral nasal prominence) along with the tissue that's part of the upper jaw (the maxillary prominence).

This is a human embryo at about six weeks of development. At this stage, these tissues should have united, forming a normal upper lip. If they do not, you end up with a cleft. What happens is that between the nostrils the tissue derived bilaterally was initially widely spaced and had a groove down the middle of it. That was going to make the tip of the nose, the center part of the lip tissue, the lateral edge of the nostril, and part of the upper jaw. Obviously, they did not unite. In order to get this kind of cleft lip the insult has to occur sometime prior to the sixth week of human development. However, the insult is usually not related to something that interfered with the last step in the union of these processes. Instead, the insult occurs in their development prior to the time that they unite. If we look at the whole embryo, almost half of its length is head and neck, because the neck ends where the arm begins. If we cut off this embryo here and tip it up to look inside at the roof of the mouth, we see the nostrils, tissue between the nostrils which made part of the upper lip, the lateral nasal prominence, and the maxillary prominence. Extending into the oral cavity from the maxillary prominence is this shelf of tissue that's going to become the secondary palate, or the roof of your mouth. It starts out as the two shelves from your maxillary prominence, which have to grow and unite in the midline. By the time you're about nine weeks old, your palate has fused down the midline if you had enough tissue to make normally sized palatal shelves. Frequently, a cleft lip is accompanied by a cleft palate. The fact that the lip didn't come together may predispose the palate to not coming together. It may be more complex than that, or you can have an isolated cleft palate or isolated cleft lip. You can have the different combinations, but one should not call a cleft lip a cleft palate unless there is one. By the time the secondary palate closes, the conceptus has reached a stage that we call the fetal stage. We recognize that we have a fetus when we can start to see ossification centers, or cartilage turning into bone. At this stage we've already established most of our major tissues and our organ systems; primarily we have to grow. After this stage, we're not going to see many malformations unless they are a result of disrupting something that has already formed normally.

Let's talk about the experimental work that's being done in abnormal development. A message I

really want to drive home is that timing is very, very critical. We've said that if there's going to be a cleft lip, an insult has to hit the embryo prior to six weeks. If there's going to be a cleft palate, the insult has to hit the embryo prior to nine weeks. Let's look at timing a little more precisely. Now you've learned embryology, you're pros and we're going to draw on that knowledge that you've just gained. You looked at embryos that were at very early stages, you looked at the face, and we recognize that these are the two halves of the forebrain. Those two halves of the forebrain should be nice and pudgy and round, almost nice hemispheres. The mouth will be here and the heart will be here. This picture shows an embryo in the lab that has a skinny forebrain. We caused this by insulting the embryo right at this time. Obviously, it's already got a defect so some things have been going amok a little bit prior to this. At this stage the heart isn't nearly as big as the one that was on Lincoln's penny, so it's prior to the fourth week. It's in the third week, and we're seeing a for brain that's much too small. We can cause that with any number of compounds or genes. It isn't specific to a particular insult. It's specific to a particular timing and a particular timing that affects a specific cell population. The cell population that at this stage seems to be very vulnerable to insult from a number of different agents, including genetic problems, are cells that are right at the edges of the neural folds, the neural crest cells, and also the cells in the very ventral midline of the developing forebrain.

This as an embryo which has been treated with ochratoxin, which is a toxic metabolite of a mold. It kills the cells in the ventral forebrain and along the edges of the brain. At a later stage in an embryo that was insulted like this, instead of having the nostrils where they should be, the part of the cells that were on the edges of the fold were subtracted. When they come together they end up making the midline. You zap your midline and you lose not only the middle of the brain, but also the middle of the face, and we end up with nostrils that are a little bit too close together. These last two examples were made by giving too much retinoic acid at a time that would correspond to the third week in a human. This one was made with ethanol at a time corresponding to the third week in human development. Here's one that probably was a result of one too many chromosomes. This one's even worse - there are no nostrils. All we have is the tissue that should have made the tissue on the sides of the nostrils. You can also see that the whole forebrain region is very, very narrow. Instead of having two cerebral hemispheres, we're going to end up with one because you separate your cerebral hemispheres by the tissue that forms from the midline. If you kill the tissue before it can make a midline, you can't separate the two hemispheres. In this type of defect, we've got two eyes but they're in one socket and they're just little bitty eyes. When the eyes form, instead of being in a normal position that would come up here above the nostril, the brain is distorted in such a way that the eyes that are developing from the brain end up coming underneath the proboscis, which is the nostril. This is known as the holoprosencephaly series, meaning a single cerebral hemisphere. It represents a range of degrees of severity down to a face that looks really rather mild. A face that one might associate with something like fetal alcohol syndrome or fetal hydantoin syndrome or a number of genetic conditions. In fact, holoprosencephaly has been linked to some specific gene abnormalities. Of particular interest for developmental biologists is one that localizes to the human chromosome number seven. It's recently been shown that on human chromosome number seven, we have a particular type of holoprosencephaly that localizes if there is a mutation there. The gene that is mutated in that spot is called "sonic hedgehog", which is derived from genes that have been described in fruit flies. These fruit fly genes are very similar to the same family of genes in the human, the mouse and all vertebrates and invertebrates. This gene is very important in establishing patterns, such as whether we're going to have a midline in our face.

A marvelous tool that's been developed within the last decade is the capacity to select a particular gene and zap it, so that you can essentially create in an animal what a mutation in that gene would cause in a human. People who have mutations in the sonic hedgehog gene have holoprosencephaly. If we can selectively interfere with that gene in a mouse embryo, for example, we should be able to create that same problem. In fact, they've been able to selectively knock out the sonic hedgehog gene, which results in getting a mouse embryo that's obviously very malformed. The malformation's not only involve the head (because sonic hedgehog is involved in more than just the head) but also the trunk and the limbs. The effect in the head is to cause a very severe kind of holoprosencephaly.

Now along comes something that is even more interesting. We've found that in order for the sonic hedgehog gene product to work, it has to be complexed to cholesterol. It doesn't function unless you have cholesterol. If we create a cholesterol deficiency, we would expect to see the same pattern of defect as the sonic hedgehog mutation. In fact, we can create a cholesterol deficiency; molecular

geneticists have done that for us by targeting another gene that's important for carrying cholesterol to the cells. This is the apolipoprotein B gene. We can create a cholesterol deficiency, although it doesn't eliminate all the cholesterol. We've found that animals that have this kind of cholesterol deficiency have a variety of defects, including open neural tubes. Instead of having the normal embryo, the neural tube is closed all along down here, and this little pit is going to be the inner ear. The neural tubes should be closed by the time the inner ear looks like this. Here's a picture of one whose inner ear is down here, but it's at the same stage and it has not yet closed this part of its neural tube. We can see where certain cells are dead or dying in these embryos; they are the ones that are around that part of the neural tube that did not close. We can get an embryo whose neural tube is wide open like this one, it should have closed way back at that earlier stage I showed you. It doesn't involve the forebrain, it involves the midbrain and the hindbrain. The same family of animals that could have a less severe genetic defect and yet we get a lot of variation in expression. For example, here's one that is even less severe, you can see a little bit of an open neural tube. Some even close their neural tube but they can also have very severe problems. This is hydrocephalus in a mouse, its head is bulging and it's not getting around too well these days. Normal mice have cholesterol levels of about 120. The heterozygotes (those that only express one abnormal gene) have a slightly depressed cholesterol level, and the homozygotes are even worse. Not all of the homozygotes appear normal, there are some that look normal. Most of the heterozygotes look normal, so how can we make all of them look abnormal?

This is a gene-environment interaction and it's the best example to show you. Here is a gene that predisposes to having a low cholesterol level, but it isn't low enough so that all the individuals are affected. Let's take a compound that we know can make the cholesterol level even lower. It is a pharmaceutical agent that was being developed to help people who have coronary artery disease lower their cholesterol levels; it also works well in mice to cause birth defects. Using our genetically abnormal animals, exposing them to this drug, can create very severe abnormalities. Before, we saw faces that were only mildly affected, looking like the fetal alcohol face. Now they have a single nostril, and some have open neural tubes. Animals that just had a moderate cholesterol deficiency before and didn't have limb defects or abnormal genitalia, now with cholesterol levels low enough, get very severe limb malformations and very severe abnormalities in the genitalia. These animals, although produced artificially by depressing their cholesterol levels, look very much like the animals that didn't express sonic hedgehog at all. There are many complex interactions occurring. Genes that are establishing patterns, through the compounds they interact with in a very specific way, at a very specific point in time, plus the cell populations that express those genes and respond to those genes.

We have some tools available to study those genes. But will those tools allow us any degree of predictability relative to the kinds of compounds with which you are concerned? In order to allow us to explore that, we've got to simplify our systems. We've got to get down to a system where we can look at known susceptible cells, with known gene expression patterns, in known vulnerabilities. We know, for example, that neural crest cells are very, very sensitive to many things at this stage in development. What we can do is take embryos, even though they are very tiny, and we can get students who are so enthusiastic that they don't care if they spend hours dissecting out little parts of these embryos. They can take off the edge of these folds, and let them sit in a culture dish for awhile and the neural crest cells, instead of migrating into the middle space in that embryo, will migrate out into the dish. They don't do real well, but they do well enough for long enough to allow us to examine them. This is a sensitive population and this is the population we have to look at most closely, the most sensitive one. We can use specific fluorescent probes to look at the receptors that are expressed on the surface of the cells and see how those receptors change in response to particular environmental agents. We can use technologies that provide us the ability to follow living cells as they respond to these toxicants. Confocal microscopy is a technology that allows us to look inside living cells using fluorescent probes to watch what's happening inside of the cells through time. For example, we can take neural crest cells exposed to ethanol in culture, and follow what happens to the levels of calcium inside of the cells. We have a lot of wonderful tools like this that allow us to get at very specific cellular levels and do dose response and time response studies in cell populations that we know we need to be examining in order to understand specific kinds of birth defects.

Birth outcomes and environmental pollution: What has been learned from studies of populations exposed to contaminated drinking water and toxic waste sites

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My talk won't have as much certainty as the last one, because we're really at an early stage in investigating the associations between exposure to environmental pollution and adverse birth outcomes in humans. Although we're at an early stage, there have been some studies done, so we can start taking stock of where we are and where we should go. The last speaker talked about how common birth defects are. I'm going to talk about how rare they are when you get down to particular defects that you'd like to study, as opposed to lumping them all together. There are a number of states that have birth defects registries that collect incidence data and link those data to environmental data such as drinking water analyses and toxic waste site information. Many of the studies I'll discuss have utilized these registries, including Iowa, California, New Jersey, New York and Missouri. However, there are many states that do not have statewide birth defects registries. Some studies involved special efforts to create registries, such as studies which occurred in Woburn, in Tucson, and in a few other locations.

If you focus on a particular birth defect that's fairly homogeneous etiologically, you generally have a small number of cases in addition to environmental exposures that are not that frequent. This can result in a lot of uncertainty in your estimates. The temptation is to start lumping cases together. For example, you might look at all cardiac defects instead of focusing on subgroups; you look at all central nervous system (CNS) defects instead of just neural tube defects. If you do that, you may find that you start obliterating any association. However, this situation may be necessary because of small numbers of both the health outcome and the frequency of the exposures of interest. Another problem inherent in these studies centers on how they define exposures. This affects the comparability of the studies. Exposure assessment can be a problem. For example, whether samples are taken at specific distances from a landfill as opposed to utilizing data from on-site samples at a toxic waste site; whether we use data from existing drinking water databases versus actually modeling a water distribution system. The studies I'll be discussing run the gamut, they look at different kinds of exposures: air pathway exposures, drinking water exposures, different chemicals occurring at different sites or in different contaminated water supplies, different levels of chemicals, etc. It's very difficult to make comparisons, but we'll try. There are also problems in environmental epidemiology related to exposure misclassification. One such problem involves mothers that move during their pregnancy. Some studies use birth certificates to determine residence, which is simply the mother's residence at the time of birth; it may not have been her residence during the pregnancy.

Two of the studies I will be talking about were funded by ATSDR - one in North Carolina and one in California. The California study, published in 1991, was conducted in five counties in the Bay Area. This study, which was done at the census tract level and involved almost 200,000 births, is an example of a data linkage investigation - they used data from a birth defects registry and from Superfund sites. ATSDR has information on Superfund sites which we use to estimate which sites may have exposure pathways. In the Bay Area study, less than 12% of the census tracts actually had a site. After adding the estimates of whether the toxic waste sites were likely to expose people, there were only about 5,000 births eligible for inclusion. Most of the toxic waste sites had limited data, so it was impossible to determine whether or not an exposure was likely. Most of you know about Times Beach, Missouri, where waste oil contaminated with 2,3,7,8-TCDD (dioxin) was sprayed on parking lots and dirt roads from 1971-1973. The levels of dioxin were around 2 parts per million. In order to give you a sense of how high that is, there was a site in Pensacola, Florida last year that had 200 parts per trillion dioxin which resulted in relocation of residences, after a lot of

public pressure. Times Beach had about 400 exposed births between 1972-1982, and about 400 unexposed births. We found small differences in birth weight and a small odds ratio for low birth weight; most of the effect was in pre-term births. It seemed logical that if there was an effect, it would be in pre-term births, but the timing wasn't right. Most of the effect occurred way after the exposure was likely. Consequently, there wasn't much effect on birth weight.

Now I want to focus on studies conducted at Lipari and at Love Canal. In the Love Canal situation, Hooker Chemical bought the Canal in 1942 and started dumping; the dumping ended sometime in 1953. The Health Department used birth certificates to identify all births that occurred between 1940-1978, and interviewed the mothers. Using maps, they determined where the natural drainage areas were, the hypothesis being that these posed the most likely exposure pathway as opposed to being just adjacent to the dump itself. Sure enough, that was the case. They found elevated odd ratios for all live births. In the Lipari study we focused on term births, looking at low birth weight and birth weight difference. Lipari is a landfill in Mantua, in Gloucester County, New Jersey, in the southern part of the state. It's a landfill that's located in one town but all the pollution goes into another town. The chemical dumping occurred between 1967-1969, the dump was closed in 1970. Everything under the sun was found on site including a lot of volatiles and pesticides, and TCDD was also found in air samples. The study went back to 1961, looking at all births within a kilometer of the site, then actually adjacent to the site. The unexposed group was greater than the kilometer distance. Adjacent to the site, there was a difference in average birth weight among term births of close to 200 grams. For births occurring one kilometer away, the difference was much lower, so it's very important to be precise in your exposure assessment. At the kilometer level, the difference was 70 grams and the odds ratio was about 1.9, so it made a big difference to focus on the immediately adjacent area. At Love Canal, the dumping and the increase in low birth weight run fairly parallel; the low birth weight only starts to diminish and come back to the state average about 5 years after the dumping ceased. The birth weight rate is mimicking the dumping, the peak is 1950, probably when the dumping was most severe. Our reference group was births occurring at greater than one kilometer from the site. At one kilometer or less the difference is about 70 grams. When we focused on people living adjacent to the site, during the period when we expected the exposures to be the worst, it's a much greater difference. So all the action is right up against the site. A lot of people say "well, could it be smoking, lower SES, or whatever," but during the other time this area is actually doing better than the reference area. It's only when the exposures were the worst that birth weights plummet, which seems to indicate that there probably was an effect; there were a lot of VOCs at this site, a lot of the surface water was contaminated.

Let's shift our focus to drinking water studies. There is a degree of uncertainty inherent in these studies due to several assumptions which have been made in estimating drinking water exposures, especially when using birth certificate information to determine the mother's residence. One study focused on Woburn, Massachusetts, which is about 10-12 miles north of Boston. There's a book called *Civil Action* which goes into the legal issues around this site, it's an interesting book. There were two wells that were opened in Woburn in the early-mid 1960's; there was a tannery and a chemical plant polluting the groundwater with trichloroethylene (TCE) up to 267 parts per billion. The people were suspicious about the drinking water, and a childhood leukemia cluster was identified in the town as a result of an investigation which was initiated due to public concerns. This cluster was located a half a mile or more from the well. Discovery of this cluster prompted testing of the well through a state program. They found the high levels of TCE and shut the well down. This occurred in 1979, but the pollution probably existed for quite a long time prior to that. The investigation looked at small for gestational age, defining it as the 10th percentile based on Massachusetts norms, focusing on births in 1975-1979 that had some exposure to these wells versus births either in East Woburn or the entire town (which was not exposed). The odds ratios vary depending on how you define exposure. A higher odds ratio was found when exposure was defined as "high", meaning the 90th percentile of probability of getting the drinking water. If you look at exposures during the third trimester vs. during the entire pregnancy, you also have higher odds ratios. We hadn't seen that result in other studies.

Another study was done at Camp Lejeune looking at PCE contaminated wells that were serving a large area of the base housing, and also at TCE contaminated wells serving only officer's housing. Again the number of cases was small; they examined small for gestational age (10th percentile). The time frame is 1968-1985, which is roughly about the time we think the exposures occurred before they shut the wells down. For PCE (births occurring within the

entire base population) the odds ratio is 1.2 with a very tiny 24 gram difference. Looking at mothers 35 years and older, they found an odds ratio of 3.9 and 200 gram difference. We don't know what to make of this. The odds ratio for TCE among male births is close to 4, with a 300 gram difference in birth weight. The subgroup seems to be showing something more sensitive than looking at it all together, we don't know why.

I want to talk briefly about the triazines study at Rathbun, Iowa, which was recently published. During 1984-1990, triazines in Rathbun reservoir averaged around 1 to 3 parts per billion. The odds ratio for small for gestation (10th percentile) was 1.9 compared to other towns in southern Iowa which did not have water supplies contaminated with triazines at those levels. Finally, in the New Jersey study, carbon tetrachloride was the only VOC that really had any impact on low birth weight, but it didn't show a big difference in average birth weight deficit.

ATSDR has funded a number of studies on birth defects: the two California studies, the Iowa herbicide study, New Jersey study, and one of the two New York studies. Back to Times Beach briefly: there were only three central nervous system defects in the population living in the exposed area, but the expected number was one. So the odds ratio was three, which was not statistically significant. Putting statistical significance aside, it's interesting given what we found among Agent Orange exposed veterans. The California study looked at census tract level and had 5,000 birth defects in the five county region of the Bay Area from 1983-1985. They took random samples, five to one of normal births, to study central nervous system defects and other birth defects. Using available data, they characterized sites by the exposure potential and found an odds ratio of 0.5 for all CNS defects, and an odds ratio of 1.9 for neural tube defects. It's very important which end point you chose, even at this level. The later California study focused on three particular defects; neural tube defects, collapsed and conotruncal heart defects. They used GIS technology to map locations down to the latitude and longitude, and looked at most of the state, eliminating parts of the Bay Area and Los Angeles. By this method, they were able to locate births to within less than a quarter mile from an NPL site, so there was a much better characterization of exposure. Again, the level of uncertainty exists due to the fact that most sampling done at toxic waste sites is on site, not off site. In this case, they found an odds ratio of 2.1 for neural tube defects.

The two New York studies (1983-1984 and 1985-1986) looked at all CNS defects. Using similar techniques (the later study employed GIS technology) and more of the sample data, neither found much in the way of increased odds ratios for all CNS, probably because they didn't focus on neural tube defects. The drinking water studies that looked at all CNS defects and neural tube defects alone found higher odds ratios, the larger odds in the high versus low exposure groups. The New Jersey study found higher odds ratios across the board for all CNS and neural tube defects. They had carbon tetrachloride greater than 2 parts per billion, and trichloroethylene in greater than 10 parts per billion in the drinking water. Not much is known about nitrate and CNS defects, but there is some indication that nitrate may be related to CNS defects. Iowa should follow up on this, using their Birth Defects Registry and nitrate data. So, based on all I've just shown you, there seems to be some indication that either drinking water contaminants or VOCs coming out of toxic waste sites might be associated with neural tube defects in particular.

With oral clefts you get a different picture. We don't see much in either the 1991 or 1997 California studies, which are based on small numbers. The New York study didn't see much going on either. In Woburn, you get a stronger relationship; all the elevation is in cleft lip. In the Iowa study, the elevations are in cleft palate; there weren't any cleft lip in the Iowa study. The New Jersey study looked at oral clefts combined and found some elevated risk with these contaminants.

For cardiac defects, the only toxic waste site studies are from California, which focused on the conotruncal defects. This study is actually very interesting. Interviews were conducted to determine where the mother resided during the pregnancy. By mapping that information they were able to get to the quarter mile exposure level. They used available environmental data by focusing on a particular defect that might be very sensitive, and they found a very strong odds ratio. That is an interesting finding to us, which we hope to follow up on. On the drinking water side, there was an interesting finding several years ago in Tucson where they had TCE in the 200-300 parts per billion range. This study was prompted by a cluster. Around the same time, there was a cardiac cluster in Santa Clara County in California where 1,4-trichloroethane was spilled. They were trying to link the cluster with the contamination from that spill. We're not sure what to make of those studies from California. In Woburn, where the levels were comparable to Tucson, we really didn't see anything. In New Jersey the levels are nowhere near

as high as Tucson or Woburn for trichloroethylene, and we didn't see much for cardiac defects. On the other hand, the Iowa Rathbun study found an odds ratio around 4.7 compared to other southern Iowa towns. Rathbun towns were at 5.7 compared to the rest of the state, so triazines seem to be something that needs to be followed up for sure, particularly for clefts and cardiacs.

With musculoskeletal defects, we don't see much. In the California study, most of the odds ratios were less than one, except for an odds ratio of 2.1 for limb reductions. The Woburn study had mixed findings. The triazines in the Iowa study seem to be related to limb reductions, so that's another endpoint that needs to be followed up.

There are disadvantages on the exposure side when conducting these very difficult studies. The exposures aren't very frequent, and the data that are available to estimate exposure are oftentimes not enough. You may have only one water sample or have only on-site samples from toxic waste sites. You never have the information you want, which makes it very difficult to estimate exposure. Even the data linkage studies are never that simple. On the outcome side, it makes a difference which endpoints you focus on. All CNS defects may not be sensitive enough, all cardiac defects may not be sensitive enough. You have to get down to the subgroupings, and the right sub-groupings. On the other hand, small for gestational age, and particular birth defects seem to be sensitive; of course, there's no latency. Where there are birth defects registries, we can actually do these studies rather easily. You can use the birth certificates for birth weight and small for gestational age studies, but you can't use them for birth defects studies. It really is important that states have birth defects registries, and utilize them for these kinds of studies.

Community right-to-know: Contaminants in drinking water supplies and reproductive health

Gina Solomon, M.D., Natural Resources Defense Council

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Since agriculture is the number one industry in Iowa, I will point out that pesticides, particularly herbicides and nitrate, can run off into water from agricultural work. Heavy industry can also produce discharges that impact drinking water. Small businesses shouldn't be overlooked. For example, PCE, or tetrachloroethylene, has been found in drinking water in numerous states including Iowa; that's the main solvent used in dry cleaning. Non-point sources such as transportation, and consumer product use are particularly difficult to control and also important. So we are talking about the whole range of synthetic organic chemicals that can end up in drinking water. That's a pretty broad bill. I also wanted to point out that endocrine disrupting chemicals, such as the alkylphenols and alkylphenol ethoxylates (used in detergents) and bisphenol A have been detected in drinking water in numerous areas. There's a study coming out this month in *Environmental Science and Technology* on detections of these chemicals in shallow wells and shallow aquifers in Cape Cod, Massachusetts.

The result is that numerous chemical contaminants can potentially end up in drinking water. The question is "Do we care?" Well, the shuttle driver that brought me here last night from the airport was telling me a little bit about Iowa City water, he was focusing on the offensiveness of the water. He said that in the past it had a sort of a greenish-brown color, it had a funny smell and it was kind of thick and cloudy. He said that it's not so much of a problem anymore, which I took to be a good sign, but he said that sometimes it tastes very strongly of chlorine. The greater concern to most of us assembled here is the potential health effects implicit in some of these things that might be in our water, or are in our water. There are a number of principles we need to be concerned about when we look at these issues.

One is that during pregnancy there are a number of changes in the body's homeostatic mechanisms, including how drugs are absorbed and

metabolized. The same thing applies to toxicants from our environment. It's particularly important to know that changes in volume can affect absorption of inhaled materials, and can impact the way our bodies metabolize or clear different chemicals. These changes could increase or decrease susceptibility, at least potentially. In addition, timing is absolutely critical. In fact, timing may be more important than dose when we look at reproductive toxicity. That's a different paradigm than the one for cancer effects or for acute toxicity from chemical exposures. This can be relevant due to the fact that contamination in water is usually not steady state, in fact there are spikes. It is important to control the overall level and try to address or control those spikes, their frequency and their occurrence, because a spike of contamination at the wrong time might be very relevant. Fetal life isn't the only critical period; I'm going to lump infants and children into the discussion here. Children per unit of body weight drink at least two and a half times more water than adults; if they are bottle fed, they consume about a seventh of their body weight in water every day. This is kind of shocking; when you put it in terms of a 70 kilogram man, that would equal drinking three gallons of water a day. Not only that, but infants and children have a greater body surface area to body weight ratio, which is important when you're talking about absorption of anything through the skin. They also have a greater respitory rate, and they breath more air per unit of body weight, compared to adults. That's important if you're talking about inhalation exposures. So if we think about inhalation, we think about pregnancy and also infants and children as being particularly susceptible, or at least potentially so.

Why are dermal and inhalation exposures important in our discussions today? A lot of the synthetic chemicals, particularly the volatiles, are absorbed not just through drinking water. If you look at the studies that have been done in this area, there's been a tendency, at least in some of the earlier studies, to ignore the fact that people are getting exposed not just from drinking water but also from showering, bathing and other activities which involve hot water. In fact, it was probably EPA's total exposure assessment methodology studies in the 1980s that really opened people's eyes to this. They were looking at chloroform exposures around the home, and found that if someone turned their dishwasher on, the chloroform levels in their kitchen air would increase significantly. Similarly, if they were standing over the sink doing dishes, their personal exposures to chlorine in air would also increase. There are some great studies in which people showered wearing rubber suits, and then showered not wearing rubber suits, and other studies where people swimming in pools either wore scuba tanks or did not wear scuba tanks to determine what proportion of exposure comes from dermal versus inhalation. Basically, it's the rule of 50%: less than 50% of what you get is from drinking water. Of the other 50%, about half is from absorption through the skin and half by inhalation. This is mostly in the shower. For a long time the assumption was that people drank two liters of water per day (which is a very generous estimate) and that was all the exposure you got from a contaminated source of drinking water. This is fine if you're talking about a metal, but not fine if you're talking about volatile organics. Another assumption is that people take one ten minute shower per day. I can tell you from experience that the person in the room next to mine took one twenty minute shower this morning, and that a lot of people take more than one shower a day, especially if they exercise during the course of the day. So one ten minute shower a day may not be a very health protective estimate if you want to incorporate it into risk assessment.

So the exposure does not come from oral ingestion alone. In addition, the physiology is different in terms of how the different routes of exposure potentially impact the human, particularly if we're talking about toxicity to the fetus. For example, when you drink something it initially gets filtered during a first pass metabolism by your liver. Toxicants may be substantially metabolized which can be important if the liver is the end organ of interest or concern; or the substance may be metabolized to something more toxic. When you inhale something or absorb it through your skin it goes directly into your systemic circulation. It doesn't go through the liver initially, and so results in a higher initial first pass exposure to the other organs in the body, and potentially to the fetus. So the route of exposure is very important.

As part of my job, I respond to questions and concerns from the public. A couple of months ago, a woman called me and said she had heard that an environmental group, not mine, was talking about a chlorine ban. She was worried about swimming and her exposure to chlorine in the swimming pool. She mentioned that she just found out she was pregnant, so she was particularly worried about whether she should continue swimming every day, as it's her routine, during her pregnancy. It was actually not such a simple question to answer even though it was asked for perhaps a naive reason. In fact, chloroform exposures have been measured in the air in and

around indoor swimming pools; biomarkers of chloroform exposure by both exhaled breath and blood markers have been measured in people after swimming. The levels are not extraordinarily high, but there is at least a potential risk there that I wanted to discuss with her. The highest numbers are from a study in which they had people swimming with scuba tanks on so that they could separate inhalation from dermal; they also found that certain factors increase absorption of chloroform after swimming. More swimmers in the water is a factor because the water gets agitated and more of the chloroform gets airborne in a layer just above the water surface which is the breathing zone of the swimmers. I think I've covered some of the issues about vulnerable windows and susceptible populations, and also some of the issues about how we get our exposure and how that might be relevant. It really is important to keep in mind how a chemical behaves in water, and how it behaves in the environment.

Volatility is obviously important when you're talking about inhalation, as is a chemical's capacity to penetrate the skin or bioaccumulate. This came up recently in California, where a number of wells and surface water supplies around the state have been found to be contaminated with ammonium perchlorate, which is a component of rocket fuel and it's also used in the manufacture of explosives and fireworks. The funny thing is that a different perchlorate salt, potassium perchlorate, was used medicinally as a thyroid blocker for many years until it was taken off the market as being too toxic due to its ability to cause bone marrow suppression or aplastic anemia. Obviously, the levels found in the drinking water in California are orders of magnitude lower than those that were used medicinally. It's interesting that we have an endocrine disruptor in water in certain areas of California. One of my initial questions was that since this is a salt, how does it behave in the environment, how does it behave in the shower, in water, does it cross the placenta? It probably doesn't penetrate the skin because it's polar. Does it end up in an aerosol phase in the shower and thereby is it potentially inhaled as well as orally ingested? Those were some of the questions I had.

Another point is that water is a complex mixture, which makes studying it very challenging. People in science like to look at one thing at a time, they like to isolate each variable. It's hard to do that in the case of drinking water. There's quite a body of research looking at quantifying water consumption as an exposure end point, looking at the toxicity in animal studies of a common water mixture. The National Toxicology Program is working on that; instead of giving each chemical individually to rats, they are creating a mixture and giving that to the rats. The Endocrine Disruptor Screening and Testing Advisory Committee is coming up with priorities for what things need to be screened and tested for endocrine disruptive effects. We're looking at commonly occurring mixtures; one of these is likely to be drinking water.

I mentioned the Cape Cod studies done by the Silent Spring Institute, where they are testing Cape Cod water on one of the screens for estrogenicity, the MCF7 Breast Cancer Cell. This is a situation where we have a lot of complex issues, a lot of scientific uncertainty. We really need to decide when any kind of action is appropriate, and if so, what kind of action is appropriate. For example, in the case of trihalomethanes we're balancing different public health goals; we're balancing the goal of prevention of gastrointestinal disease with the risks of some of the disinfection by-products. In these situations it can be hard to say "we need to ban this" or "we need to regulate clearly to this level or that level". Instead, I think we should emphasize the need to communicate the uncertainties, communicate what we do know and try to involve the public in the debate. In clinical medicine, for example, we frequently operate with a considerable amount of scientific uncertainty. In those situations, we are accustomed to talking with patients and their families, explaining the uncertainty, bringing into the equation their priorities and their wishes, and then making a decision. There is an established way of involving the public in decision making in the setting of scientific uncertainty. That's something we're going to have to move towards more and more. As we said this morning, absolute scientific proof is a holy grail which may be impossible to achieve. If we wait for absolute scientific proof, we're never going to get anywhere; we're going to end up constantly holding back and then being buffeted by political pulls in the other direction.

Let's talk about some of the different right-to-know issues: there's right-to-know on health outcomes, and there's right-to-know about exposures or right-to-know about issues that are potentially relevant to human exposures. As you know, "exposure" is really a whole pathway. Issues that could be relevant to human exposure start with the production and sales of a specific chemical. If it's not produced or sold in significant quantities, exposures are unlikely, whereas if it's produced and sold in large quantities, exposures are more likely. Next, information on chemical releases is potentially

pertinent to human exposures - information on levels and environmental media, and information on levels of human intake, which is often difficult to measure. Exposure is technically defined at the outside boundary of the human body. As soon as it crosses that boundary, you're being exposed. Once it has crossed that boundary and is inside the human body, there's also the potential for biomonitoring or looking for biomarkers for exposure. The whole pathway of exposure is not really a direct pathway, because some of the things in the earlier phases never end up in people, and people are probably exposed to more than is actually measured in biologic tissues because they are constantly metabolizing and excreting.

Right-to-know is mostly governed by a series of laws which require manufacturers and employers to disclose information about toxic or potentially toxic substances. The disclosure could be to workers, to the community or to governmental agencies. I want to talk about the major right-to-know in the workplace which is the OSHA Hazard Communication Standard promulgated in 1982 and revised a decade ago. Material Safety Data Sheets are right-to-know about hazards; they contain information about the potential health effects of a chemical, whereas information on chemical labeling, or exposure records from monitoring in the workplace are obviously exposure measures. The Superfund Reauthorization Act of 1986 established the Toxics Release Inventory; people have often focused on this as the most useful source of exposure data. I actually don't think that's true; it has perhaps been the most accessible and the most intuitively understandable to the general public because it reports pounds of stuff released to air, water or land. We may in fact be only moderately concerned with that, but people have tended to focus on those issues. More pertinent to water issues, the Safe Drinking Water Act reauthorization established a requirement for consumer confidence reports, which are annual notices with the water bill describing what has been detected from monitoring drinking water supplies and water systems. There's also a requirement for rapid and prominent publication of any violations of standards. This is an estimate of potential human intake, so it's an exposure right-to-know law. In certain areas, including Iowa, historical information is collected in a centralized place, so it's not cross sectional, or from one point in time. Rather, these databases contain broader sets of contaminants over broader periods of time, which is more useful for research purposes, and may be more relevant for certain kinds of questions from the public. There does need to be a translation step, as far as making the information clear, understandable and putting the levels in some kind of context.

California Proposition 65 prohibits contaminating drinking water with any chemicals that are known by the State of California to cause cancer or birth defects. For non-water discharges, there's a requirement to warn people before exposing them to such chemicals. There are approximately 500-600 chemicals listed as known to the State of California to cause either cancer or birth defects, so this has imposed quite a lot of obligation on industries and others to warn California consumers. In the case of consumer products it's had quite interesting effects. There have been a number of situations in which products have been reformulated rather than carry a warning. This is using the power of the market to deselect more hazardous products and select potentially less hazardous products. The drinking water aspects of the law have been much harder to enforce than anticipated because you have to pin the discharge on a specific discharger; by the time something's in water it's often hard to figure out who put it there. In addition, since government agencies are exempt, many water systems are exempt from the law.

One of the interesting things that is being done more often is localized research, which involves getting a historical and deeper cross-sectional map of a specific area. Iowa is one of the places that's been on the forefront of this. There has been a long-term effort to collect information that allows us to put together an exposure picture and a health effects picture from the surveillance data and the monitoring data that have been collected. This is going to be really important as we begin to look at complex mixtures, and as we look at exposures coming through various media - air, water, workplaces, etc. In addition, biomonitoring and biomarkers of exposure and effect will be extremely important as we look at more subtle endpoints.

Structural birth defects may be fairly obvious in many cases, although there are problems with reporting. Functional birth defects have really slipped through the cracks, and they are extremely important. I.Q. point deficits, behavioral problems, or similar deficits may be getting missed because they are too subtle for our crude analytic techniques. A move to look more closely at those kinds of things is going to be extremely important. If you monitor exposures over time, you can set goals. For example, you can say the levels of atrazine in our drinking water are excessive at certain times of the year, so let's do something to make them less high next year, let's get

them down by X percent in five years. It allows regulation to have a focus and a purpose rather than just constantly playing catch up.

We also need more flexible scientific tools. I'm referring to the fact that if you look at statistical significance, you're going to miss a lot. In many cases we're talking about rare endpoints, we're talking about random misclassification of exposure which will tend to result in finding less of a risk than there actually is. If you hold yourself to achieving a certain arbitrary statistically significant endpoint, you may be missing things that are quite relevant from a human health point of view. This is why it's important to look at consistency of the evidence, to look at trends towards significance, and to look at point estimates. When you take into account what the weight of the evidence is showing and at what point we might want to decide that the picture is clear enough that we might want to do something. Lastly, we must keep the public involved in the process. It will pay off in the long run, as far as having a more educated public that is able to participate in decision making.

Evaluation of gene-environment interactions as risk factors for adverse reproductive outcomes

Paul A. Romitti, Ph.D., University of Iowa

Dr. Romitti is an assistant research scientist who holds appointments in the Department of Pediatrics and the Department of Preventive Medicine and Environmental Health at The University of Iowa. He has a B.S. degree in biology and a B.A. degree in chemistry from Iowa State University and a Ph.D in epidemiology from The University of Iowa. Dr. Romitti is an investigator with the Iowa Birth Defects Registry and the Iowa SEER Cancer Registry. He has a wide range of research interests including environmental epidemiologic studies, genetic epidemiologic studies and validation studies.

Adverse reproductive outcomes encompass several events. Preconception outcomes include sexual dysfunction, sperm abnormalities and subfecundity. Postconception outcomes include early fetal loss, intrapartum death, birth defects, childhood morbidity and childhood malignancy. There have been investigations of genetic and/or environmental risk factors for many of these outcomes; however, investigations of genotype-environment interactions have primarily been limited to birth defects. As such, I will focus my talk on evaluation of genotype-environment interactions for birth defects, although the approach that I will describe would be applicable to other adverse reproductive outcomes.

To explore genotype-environment interactions for birth defects, there are three main aims one would need to implement. In discussion these aims are rather straightforward. In practice they are rather complex to implement. Initially, one needs to identify children with birth defects via a surveillance system. Next, one needs to collect biologic specimens and exposure information from case and, depending on study design, control families. Lastly, once these data are collected, one needs to assess the risk of birth defects due to genetic factors, environmental factors, and genotype-environment interactions.

There are two main types of surveillance systems – active surveillance systems and passive surveillance systems. An active surveillance system entails identification of cases by trained personnel who systematically review records in hospitals, clinics and other facilities. A passive surveillance system entails identification of cases from vital records or medical reports submitted by staff in hospitals, clinics or other facilities. In Iowa, birth defect cases are identified using an active surveillance system, the Iowa Birth Defects Registry. The Registry, established in 1983 by a legislative mandate, was initially funded, and continues to be funded, by a cooperative agreement with the National Centers for Disease Control and Prevention (CDC). The goal of the Registry is to collect population-based data on all Iowa live born infants diagnosed with a birth defect in the first year of life and also stillbirths and aborted fetuses diagnosed with a birth defect. To accomplish this, multiple, overlapping data sources, including medical and vital records, are reviewed.

Once children with birth defects are identified via a surveillance system, the next step is to collect biologic specimens and exposure information from case and control families. Biologic specimens are typically collected by either venipuncture or finger prick blood collection or by buccal cell collection. Environmental exposure information is typically obtained by either in-person interview, telephone

interview, mailed questionnaire or some combination of the three instruments. In Iowa, there are three, large ongoing studies of birth defects. One is the Iowa Orofacial Cleft Study, which is a project within the University of Iowa Craniofacial Anomalies Research Center. The other two studies – the Birth Defects Risk Factor Surveillance Study and the National Birth Defects Prevention Study – are multi-state collaborations headed by the CDC, and each was discussed this morning by Michele Lynberg. Since the Iowa Orofacial Cleft Study is the longest in tenure and the most advanced in terms of data collection and analysis, I will present our experience with investigation of genotype-environment interactions in this study only.

To review, orofacial clefts are a common congenital malformation. These defects have a birth prevalence rate of 1-2 per 1,000 births with variations by race and ethnicity; rates are highest for Asians and Native Americans, lowest for African-Americans, and intermediate for Caucasians. Cleft phenotypes that we are interested in studying are cleft lip, with or without cleft palate (CLP) and cleft palate only (CP). Each of these phenotypes may present with or without additional malformations. Cases presenting with additional malformations are classified as syndromic, and those presenting without additional malformations are classified as nonsyndromic. This latter group of cases are suspected to have a multifactorial etiology; that is, several genetic and environmental risk factors.

As mentioned, the Iowa Orofacial Cleft Study is a project within the University of Iowa Craniofacial Anomalies Research Center. The goal of the Center is to identify genetic and environmental risk factors for orofacial clefts using a multidisciplinary collaboration which includes basic scientists, clinicians and epidemiologists. For the Iowa Study of Orofacial Clefts, cases are identified by the Iowa Birth Defects Registry and controls are selected from Iowa birth tapes. Exposure information is collected by telephone interviews or mailed questionnaires and blood or buccal cell specimens are collected from index children and birth parents. Biologic specimens are collected from the three family members, because our search for potential genotype-environment interactions begins with the evaluation of candidate genes. Following this, is the evaluation of risk associated with parental (particularly maternal) environmental exposures both independently and in combination with specific genotypes. Potential environmental risk factors were divided into five groups: family history, medical history, nutrition, lifestyle and occupation. This classification prompted the need for multidisciplinary teams of experts to assist with identification, measurement and analysis of each group of risk factors.

Returning to candidate genes, previous animal and human studies have identified several candidate genes for nonsyndromic CLP and CP. Among these are the homeobox gene, MSX1, and the transforming growth factors alpha and beta 3. Methods we have used to evaluate candidate genes in the Iowa Orofacial Cleft Study include mutation searches to identify polymorphic markers in each gene and genotyping of case and control specimens on polyacrylamide gels for each marker identified. Team members who have participated in these analyses include developmental biologists, molecular geneticists and molecular epidemiologists.

Family history is another important risk factor for nonsyndromic CLP and CP. To evaluate this factor, we first consider the potential modes of inheritance. One that has been identified is the multifactorial threshold model in which the combined but modest effects of several genetic and environmental factors contribute to the development of nonsyndromic CLP or CP. Another is the presence of a major gene for either cleft phenotype that is modifiable by various teratogens. Data collected for evaluation of family history as a risk factor for nonsyndromic CLP and CP are a comprehensive reproductive history for birth mothers and birth defect diagnoses for two generations of parental relatives. Team members who have participated in the collection and evaluation of family history information include clinical geneticists, genetic counselors and genetic epidemiologists.

A third group of risk factors for nonsyndromic CLP and CP are those associated with maternal medical history. This group encompasses chronic diseases such as diabetes, acute conditions such as flu or fever during pregnancy, or medications taken for certain conditions such as anti-seizure medications for the treatment of epilepsy. Data collected for the evaluation of maternal medical history as a risk factor for nonsyndromic clefting are a pre-pregnancy lifetime medical history and prenatal care and medication use during pregnancy. Team members who have participated in the evaluation of maternal medical history include obstetricians, nurse practitioners and pharmacoepidemiologists.

In recent years there has been considerable interest in maternal nutrition as a risk factor for birth defects, particularly the relationship between use of folic acid and the reduction of neural tube defects. Maternal nutrition appears to be an important risk

factor for nonsyndromic CLP and CP; suggested are a deficiency of folic acid, an excess of vitamin A and a deficiency of zinc. Data collected for our assessment of maternal nutrition as a risk factor for nonsyndromic clefting are a vitamin and mineral supplement profile and a food frequency questionnaire for the first trimester of pregnancy. Team members who have participated in the evaluation of maternal nutrition include nutritional epidemiologists and research dieticians.

A rather large category of risk factors for nonsyndromic CLP and CP is maternal lifestyle. Included are behaviors such as alcohol consumption and cigarette smoking, as well as exposures during activities of daily living such as drinking water contaminants. Data collected to evaluate maternal lifestyle as risk factors for nonsyndromic clefting are a risk behavior profile and a maternal residency history. Frank Bove mentioned in an earlier talk the importance knowing where the mother lived during the entire term of her pregnancy in order to adequately risk due to drinking water contaminants. Team members who have participated in the evaluation of maternal lifestyle include psychologists, environmental epidemiologists and reproductive epidemiologists.

Since the number of reproductive age women entering the workforce is increasing each year, there is considerable interest in the evaluation of maternal exposure to occupational agents as risk factors for nonsyndromic CLP and CP. Risk factors that have been studied include agricultural chemicals, heavy metals and solvents. Data collected to evaluate maternal exposure to occupational agents as risk factors for nonsyndromic clefting are a comprehensive occupational history, as well as a hobby and activity profile. Team members who have participated in the evaluation of maternal occupational exposures include industrial hygienists, occupational physicians and reproductive epidemiologists.

Once a surveillance system has been established to identify children with birth defects and biologic specimens and exposure information have been collected from case and control families, the next step is to assess risk for birth defects associated with genetic factors, environmental factors and genotype-environment interactions. To accomplish this, one typically calculates a risk ratio due to a genotype alone (Rg), a risk ratio due to an environmental exposure alone (Re), and a risk ratio due to the joint effect of each exposure (Rge). These ratios, Rg, Re and Rge, may conform to one of three simple models or to a more complex biologic relationship. In the Type 1 model, neither the genotype nor the environmental exposure alone produce an increased risk of disease (Rg=1; Re=1); however the joint effect of each exposure can produce an increased risk of disease (Rge>1). In the Type 2 model, the genotype alone does not produce an increased risk of disease (Rg=1), although the environmental agent alone or the joint effect of each exposure can produce an increased risk of disease (Re>1; Rge>1). In the Type 3 model, the genotype but not the environmental exposure alone can produce an increased risk of disease (Rg>1; Re=1), and the joint effect of each exposure can produce an increased risk of disease (Rge>1).

I will now take you through an example of a genotype-environment interaction that we have identified in the Iowa Orofacial Cleft study. This involves a diallelic marker in the untranslated region of the transforming growth factor beta 3 gene or TGFB3 5'UTR.1 and the environmental exposure, maternal cigarette smoking. Infants who carried two copies of the common 1 allele for TGFB3 5'UTR.1 compared to those who carried one or no copies of the allele were not at increased risk for nonsyndromic CLP (Odds Ratios (OR)=0.9; 95% Confidence Interval (CI)=0.5,1.8) or CP (OR=0.8; CI=0.3,2.2). With regard to maternal cigarette smoking, risk for nonsyndromic CLP was modestly elevated for each level of smoking evaluated (1-9 cigarettes/day: OR=1.2; CI=0.6,2.1; ≥10 cigarettes/day: OR=1.3; CI=0.7,2.3), whereas risk for nonsyndromic CP increased with increasing number of cigarettes smoked per day (1-9 cigarettes/day: OR=1.5; CI=0.6,3.3; ≥10 cigarettes/day: OR=2.3; CI=1.1,4.6). Examination of the joint effect of infant genotype for TGFB3 5'UTR.1 and maternal cigarette smoking revealed that risk for each cleft phenotype was most elevated for infants who carried two copies of common 1 allele and whose mothers smoked ≥10 cigarettes/day (CLP: OR=2.3; CI=1.1,4.7; CP: OR=3.4; CI=1.3,8.4). This finding is among the first statistical evidence of a genotype-environment interaction between TGFB3 and maternal cigarette smoking. Our data suggest that this interaction would fit a Type 2 model. With the Type 2 model, the genotype for TGFB3 5'UTR.1 alone does confer an increased risk of nonsyndromic clefting, although maternal cigarette smoking alone or in combination with the genotype does confer an increased risk of nonsyndromic clefting. Note that the Type 2 model has also been shown to explain the association between the debrisoquine hydroxylase genotype, cigarette smoking and the development of lung cancer.

Following identification of a statistical association for a genotype-environment interaction, ideally, there are two additional analytic steps one needs to conduct. First, since there are potentially multiple genetic and environmental risk factors for nonsyndromic clefting, one needs to evaluate the influence of other potential risk factors on the identified statistical association. In the Iowa Orofacial Cleft Study, control for infant gender and family history did not materially influence the odds ratios found for TGFB3 5'UTR.1, and similarly, control for maternal medical nutritional and lifestyle risk factors did not appreciably alter the odds ratios found for maternal cigarette smoking. The second analytic step is to evaluate the quality (i.e., validity) of the exposure data. An example is the evaluation of recall bias. This bias can occur when case mothers recall exposures more thoroughly than control mothers. Since the Iowa Orofacial Cleft Study is a retrospective study and the birth outcome is known prior to data collection, case mothers, in their desire to search for the "cause" of their child's defect, may more accurately recall exposures than control mothers who do not have a comparable stimulus; thus, the statistical associations identified may be due to recall bias rather than a biologic effect. To evaluate the potential for recall bias in the Iowa Orofacial Cleft Study, maternal self-reports of exposures were compared with "gold standards" which included medical records, vital records and relative self-reports. For example, in the family history questionnaire, mothers were asked to provide information on birth defect diagnoses for two generations of parental relatives. Subsequently, a random sample of relatives was contacted and asked to document their birth defect diagnoses. In essence, we compared mothers' informant reports with the relatives' self-reports to assess the quality of the family history information obtained from mothers.

Once the data are collected, analyzed and evaluated, and ideally when these associations have been replicated in other populations, a future analytic step is the identification of biologic mechanisms underlying the identified statistical associations. Kathy Sulik has already presented a very nice overview on the use of animal models to identify genetic and environmental risk factors for birth defects.

In summary, the investigation of genotype-environment interactions for adverse reproductive outcomes requires multidisciplinary teams. These teams provide expertise for identification, measurement and evaluation of genetic and environmental risk factors. In addition, these teams, along with the appropriate animal studies, can provide expertise for investigation of biologic mechanisms underlying identified genotype-environment interactions.

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Featured Speaker

Environment, health, and the future

Tom Sinks, Ph.D., National Center for Environmental Health

Dr. Sinks is associate director for Science at the National Center for Environmental Health (NCEH) at the Centers for Disease Control and Prevention in Atlanta where he works in formulating science policy. He is an epidemiologist trained in both occupational and environmental health. He received his Ph.D. in preventive medicine from Ohio State University. In 1985, Dr. Sinks joined the Epidemic Intelligence Service (EIS) at CDC. While with CDC, he has worked with the National Institute for Occupational Safety and Health and with NCEH.

I would like to start off by saying a couple of things about the National Center for Environmental Health at the Centers for Disease Control and Prevention, what we do and what our role is there. CDC is really an applied research organization and our primary constituents tend to be State Health Departments. We're different from the National Institutes of Health which do more basic research, although some of it is applied. NIH's major constituents tend to be academic universities. Our Center has about 400 people and we have a budget of about 100 million dollars. About two-thirds of that Congress gives us and the rest comes from other agencies for us to help them do their work. We operate as a non-regulatory agency. I think trying to understand what the government does in the

environment becomes extremely complex. I'm learning every day how the federal government is organized in terms of its response to environmental health issues; one could only characterize it as very fragmented. Most people think of the Environmental Protection Agency, but the Department of Energy is the largest environmental health agency in terms of numbers of people involved in the country.

CDC's role is actually very unique. We basically do our work through what we call a public health model, we don't do risk assessment. Our mission has four parts: first, we try to identify problems that exist in human health. CDC is well known for its ability to respond to emergencies under crises. Our center, which is a moderate size center at CDC, is the second most active center at sending people out into the field to do epidemic assistance in the states. We do all kinds of things that you wouldn't normally think of as CDC. We also do surveillance. We track trends of disease over time and evaluate how interventions work. We also do epidemiology to identify preventable risk factors and then develop and implement interventions. That's a little different than the risk assessment paradigm, but it suits us well.

I'm going to talk about three priorities we have at the present time. NCEH is really an eclectic group; we have programs ranging from the vessel sanitation project to a radiation program. The areas that I want to focus on tonight are biomonitoring, asthma, and water issues including endocrine disruptors. Let's start with water. We're extremely concerned with what is in our water. A very interesting report from the Environmental Working Group came out about the time the Safe Drinking Water Act was being passed; the issue was herbicides and municipal water systems. A couple of years ago we recognized how fractioned the environmental health response was around drinking water. A subcommittee of the environmental policy committee, including our center, NIEHS, NCI, and EPA, came together and wrote a document which characterized the roles of the different agencies in response to drinking water concerns dealing with human health priorities. This includes data systems for health events and data systems for exposure. Most of our efforts tend to be in surveillance of health effects. I think we need to be doing more on surveillance of exposure as well as etiologic studies.

Another thing CDC does is respond to emergencies. We were involved several years ago in a fluoride overfeed in Hooper Bay, Alaska, that resulted in one death and one hospitalization. CDC is constantly involved in epidemics of waterborne diseases, where we work with our state partners and other federal agencies like EPA. Another area that is important is evaluating the effectiveness of our prevention strategies, including regulation. Once a regulation is passed, we need to evaluate whether it has been meaningful in terms of human health. Finally, we want to protect persons who are not covered by the Safe Drinking Water Act. As a result of the midwest floods that occurred in 1993, we had the opportunity to do some private well water work in the states that were affected. We developed a sampling grid within every ten miles across these states and sampled private wells for coliforms, atrazine, and nitrate. We worked with all of the states. Here are the results for nitrate, for wells that are greater or equal to 10 mg/L. In Iowa, 20% of the wells which were randomly sampled had nitrate levels higher than 10 mg/L NO_3-N. For coliform the percentages were 60% - 80% across these states. In Iowa it was 51%. This doesn't mean that there is necessarily a health problem, but it certainly means that this bears scrutiny and follow-up in terms of the health of people who get their water from these private wells. Another thing that we're doing in terms of water is working on surveillance for health effects. We're currently funding a number of places, including Iowa, to do birth defects surveillance. There was a discussion this morning about working collaboratively across these states on some case-control studies.

I'm going to turn to endocrine disruptors. This is obviously one of those issues where you could line scientists up on either side of the room and they would be shooting at each other; somewhere in the middle is the truth. There are two points that I want to make regarding our priorities. The first is we need to put credible science on to some of the observations that are made, especially the human health observations. We're talking about trends in sperm counts and other types of health trends. We need to follow those up and determine whether those trends are real, and if they are real, are they preventable? The second priority is biomonitoring and the importance of determining what humans are exposed to in terms of some of these substances. I'll give you an example of how some of this health information is being used. A study published three months ago in *Pediatrics* basically identified the ages at which young girls go through different stages of puberty. The authors did not use this study to suggest there was a change in trends over time, but used it to define standards for clinicians and pediatricians who were practicing, to give them some reference of what normal may be. At one of the Endocrine Disruptors

Screening and Testing Advisory Committee meetings, we heard testimony from the public stating how this demonstrated that there was a change over time in these pre-pubertal stages. I would say this clearly needs follow-up, but to take this information and translate it as fact is extraordinarily dangerous. A study will come out this month from the CDC on hypospadias trends. This is a study from our birth defects registry which demonstrates that hypospadias has been increasing the past two decades and it's a statistically significant trend. This is probably not a diagnostic phenomenon, but in fact includes an increase in trends in severe case of hypospadias. Again, the warning is we shouldn't be translating this as "there is something in our environment, some particular chemical that is necessarily causing this". This is where we really need to be focusing our attention, on looking at these data and seeing what they actually mean.

I want to point out a study on the estrogenicity of resin-based composites in sealants used in dentistry. This is a screening test that demonstrates that these compounds, such as bisphenyl A, have estrogenic properties when put into cell culture. This is a very good example of where the environmental issues come into direct conflict with public health. Many of you have children who have gone through this procedure; dental sealants are the second most important tool dentists have to prevent the most common chronic disease in this country, dental caries. This, along with fluoridation of the water supply, has dramatically improved the oral health of our country, and has probably saved billions of dollars. The Surgeon General's *Year 2000 Goals* include an incentive for the Public Health Service to increase the percentage of children who get dental sealants. The issue here is, do people get exposed to bisphenyl A through this process, and is that causing a health effect? If it is, we clearly want to prevent that from happening. At the same time, we have to balance the benefits from using sealants and keep that in mind.

Other issues not related to endocrine disruptors include EPA working on a risk assessment for methyl mercury. Mercury's major route of exposure to people is from fish. From my perspective, our goal ought to be less mercury, more fish. The literature shows that people who eat high levels of fish have lower mortality due to cardiovascular disease. I think we need to be very cautious when we're dealing with mercury in fish. Do we send out a message that people ought not to eat fish when we know fish itself is a highly nutritious source of protein, and is very economical? And yet how do we deal with this difficult issue of mercury? These are very complex issues. Another issue that will be coming up is dioxin. The most common source of dioxin exposure for people is breast milk; dioxin levels in breast milk. We have to be very careful about sending a message to women not to breast feed due to background levels of dioxin, when we know that breast feeding is very important to the health of infants. In fact the *Year 2000 Goals* for public health are to increase the number of children being breast fed for longer periods of time. So how do we balance these things? This is the type of role where public health and the environmental regulatory agencies have to come together and address these difficult questions.

The NCEH Environmental Health Laboratory has scientists who are experts in measuring toxicants in people. Biomonitoring helps us characterize what toxins get into people, and how much gets into people. It helps epidemiologists characterize exposure better so there is less misclassification, and we can conduct studies more accurately to determine if disease is related to exposure. Many environmental epidemiologic studies are hampered by poor exposure assessment; exposure assessment is extremely important to doing good science in this area. It helps us determine what populations are at increased risk, helps set priorities, and has been used to demonstrate how effective prevention strategies are. For example, taking lead out of gasoline.

Here are some numbers that demonstrate work we did in connection with NCI, NIEHS, and EPA on the *Agricultural Health Study*. We did a pilot study of Iowa farmers and North Carolina farmers and looked at their levels of atrazine exposure on the farm. Seventy-three percent of the farmers and 53% of their children had detectable levels of atrazine in their bodies. This is a very small study but the fact is that we can measure these types of things in people and it gives us very important information. We're proposing to measure several endocrine disrupting compounds in the next National Health and Nutrition Examination Survey (NHANES-4). We hope to be able to measure many of these compounds and have a snapshot of what's going on in the population in terms of exposures. The other area where we ought to be looking in terms of measuring exposure and possibly health effects related to endocrine disruptors is newborn screening. Newborn screening was first applied on a population in 1961; now, 48 out of 50 states have legislation that mandates newborn screening. The other two states have the authority to carry it out. The importance here is that we have a very good opportunity to collect information for surveillance purposes which is inexpensive, easy to

store and we may be able to measure a lot of things with respect to exposures. The NCEH lab does the national quality assurance on newborn screening for at least ten of the genetic tests. The issue we have to deal with in using tools like this is that technology is changing so fast. Any biologic materials that we collect present an opportunity to do genetic testing. Newborn screening is a wonderful opportunity for us to get genetic banks of information on people in this country. The limitation of using this information has to do with the risks of genetic testing. Until we as a society pass laws that preserve the rights of the people being tested, we will not be able to use this technology very well on a broad scale.

Our third priority involves a huge opportunity for public health, and one that hasn't been addressed yet. This conference isn't talking about it and that's asthma. Asthma is one of those issues that could be chronic disease, it could be environmental health, but whatever it is we're losing the war. Again, the *Year 2000 Goals* for asthma say we should be decreasing the incidence of asthma attacks and the prevalence of asthma by the year 2000. Goals include reducing hospitalizations, reducing the number of children exposed to environmental tobacco smoke (which we're probably doing), and reducing the proportion of persons with asthma who experience disabilities. We're losing this war; of all the goals we've set for improved health, asthma is one we're falling very far behind on. From 1984-1994, prevelance rates increased dramatically. We did a survey of the states to determine their interests in doing asthma control programs. Forty-eight of the fifty states responded and about 80% said they were extremely interested in conducting asthma-related work.

One problem we have with asthma is that there really are no good data. Asthma surveillance data currently are limited to mortality statistics; in some areas you may be able to find hospital discharge diagnoses. Otherwise you have to go conduct special studies to get data. Two months ago I represented the department for the Health and Human Services response for the ozone PM_{10} *(editors note: PM_{10} = Particulate Matter with a nominal size of 10 micrometers in diameter)* standards. EPA is going to set a new standard for $PM_{2.5}$; there will be millions of dollars set aside to get that monitoring system going before there are any decisions about what areas are within compliance or not within compliance. That information will help determine whether or not there's a problem relating to $PM_{2.5}$. One problem is that there is no similar investment going on in terms of surveying the health effects that might be related to PM: chronic respiratory disease, asthma, and of course mortality data.

Without the investment on both the health side and the exposure side, we're never going to know how effective our interventions are. We need to be able to measure the background of what it is we're trying to intervene on. I think asthma is a wonderful candidate. We know we have a tremendous problem; we're losing the war on asthma. We know it's a multi-bacterial illness that's probably related to both ambient air pollution and indoor air pollution, and there's a wonderful opportunity to work with EPA if we had the resources to do that. We would like to work with the states in terms of education, surveillance of asthma, and developing community asthma interventions in applied research. Within these priorities, our Center is bombarded by the emergency of the day; such as thyroid cancer from the Nevada test sites. In that mix of being barraged by the emergency of the day and the various programs that we develop, we still try to develop these programs which hopefully will improve public health.

Session 3: Endocrine Disrupting Chemicals

Estrogens, genes and development

John McLachlan, Ph.D., Tulane/Xavier Center for Bioenvironmental Research

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My talk is about some ideas we have been struggling with for the last 20 years. First, I'll discuss some approaches we have taken to see how the environment may be interacting with a variety of biological systems up and down the cytogenetic scale. As we studied this, we realized that some of these principles apply to both plants and humans. In 1975 I published a paper in *Science* describing a study where we sex-reversed male mice by giving their mothers the potent estrogen DES. About four or five years ago, we published a paper where we sex-reversed turtles by painting their shells with some estrogenic chlorinated hydrocarbons. I was amazed at this whole idea of sex reversal until I got to New Orleans and realized it is really no big thing.

Fundamentally, what I'll be talking about is the way the chemical environment interacts with biological systems that can lead to disease and dysfunction. Environmental health researchers have spent a lot of energy and time looking at the ways chemicals interact with genetic materials. Strategies have been used to look at mutations, different diseases, dysfunction and cancer. Endocrine disruptors, environmental hormones, or environmental agents can have effects working through signal transduction systems in which there are no permanent changes that we can measure as easily as DNA adducts, or mutations that are stable over generations. We have a whole new challenge to understand how the environment can work through signaling systems that in some cases lead to disorders that may be long-term. They may have been caused by a very short exposure to a low dose of a compound or several different compounds working together in a variety of different ways. This whole area can be described as environmental signals over a broad spectrum of environmental hormones or steroid hormones. These compounds in the environment can mimic or alter normal biological signals or corrupt these signals. We have been studying these exogenous signals over the last 15-20 years. These exogenous signals can actually alter the signals that go on within all of us. The signaling systems really include three main integrated systems in the body - the endocrine system, the immune system, and the nervous system. We now know that these integrated systems are themselves integrated across other systems, so the principles I'm talking about to illustrate the endocrine system could be used with these other systems as well. Finally, I will discuss data that describe signals that go on between organisms and between species, and how the environment may alter some of those signal pathways.

One of the most important things to keep in mind is that biological signals are common in most vertebrates. Hormones, neurotransmitters and other biomolecules essentially integrate actions from our brain throughout our system from a variety of different glandular organs that essentially keep us in homeostasis. When we talk about environmental signaling, we are looking at compounds in the environment that can either block or mimic these endogenous signals. These can be a wide variety of a different compounds, chemical structures, and forms. Some of the same principles I am talking about with respect to chemicals have been shown to work with changes in temperature, as well. There are a variety ways we can look at this which gives us a heuristic approach to understanding changes that occur environmentally that we don't know how to explain otherwise.

One of the things that has helped us in our research is the knowledge that environmental signals can mimic hormones or block hormones. If we know how hormones work, perhaps we have a route to understand how different compounds may work, and we may have ways to assess them. One approach I took was published in a short paper 4-5 years ago called *"Functional Toxicology"*. The idea was that

these compounds in the hormone site would have to have a receptor. This is not totally the case, it is more complicated. In a simple example, you can imagine an environmental agent (toxicant A) interacts with a hormone receptor and reduces the complete hormonal response. That would be a hormone-mimicking compound. In another example, another environmental agent (toxicant B), under different physiological situations, will block the interaction of an endogenous hormone, then you have a hormonal block. Toxicant B might be tamoxifen, an anti-estrogen, which is used to treat breast cancer by blocking the interaction of the natural estrogen receptor. This is one way we can utilize what we know about the mechanisms of forward action to try to understand how environmental chemicals might interact at a different level.

Our lab put together a system in which we transformed normal yeast cells with human genes to express the estrogen receptor by putting plasma in. You have a reporter construct which codes to the beta galactacydase gene and the estrogen response element, which is a piece of DNA that recognizes the estrogen receptor when it is in a form binding in estrogen. That will then turn on this reporter construct in the yeast. The beta galactacydase will catalyze an enzymatic reaction, which results in a colormetric change. Blue patches appear in the yeast dish, so you can essentially screen petri dishes for thousands of colonies of yeast. This is a fairly quick way to take advantage of the estrogen receptor at the functional screen.

One of the things we decided to do was ask questions about the biology and pharmacology of these compounds. We took advantage of this very good estrogen responding in vitro system and asked a question about the proteins in blood that are known to carry these hormones. Do they carry environmental compounds the same as natural estrogens? It is well known that estradiol binds extensively to sex hormone binding globulin where other potent toxicants do not. In fact, many people think that is the reason DES is so phenotoxic in humans - it is not sequestered outside the cell as natural estrogens are. The approach in this particular experiment was to take our yeast cell system expressing the human estrogen. Estradiol reporter in the body is a serum protein and will be kept outside the cell to some extent, so you get a certain dose response. DES will go right inside the cells. The question we asked is "are any of these ecological estrogens like DES or like estradiol?" Would we get a bluer plate or a less blue plate (as in the simple experiment) by giving more and more serum?

In the laboratory, we added exogenous and natural estrogens to sex hormone binding globulin to see what the effects might be. With estradiol, as we added more and more protein, the signal was damped down so when you arrive at normal serum levels of sex hormone binding globulin, you are down over 50%. Whereas with DES (as we knew from previous studies) there is very little change. Some of these other environmental chemicals, such as kepone and DDT, are more than DES and are not as routine. Therefore they gain access to the cell much more effectively. Here is an alligator serum slide. The left hand panel is human serum; you can see the human serum damped the signal from estrogen whereas the DES and environmental agents were not damped. In the alligator serum there is actually a greater discordance between the natural estrogen and the synthetic environmental estrogens with this particular species. This led us to develop a large collaborative study with the New Orleans Zoo, where staff is collecting blood from every species in the zoo. This has caught the attention of a number of other zoos across the United States, which are now collecting blood samples from various species for use in the experiment. One possibility is we might pick up differences that follow different biogenetic lines. We are asking the question "how might herbivores, carnivores, and omnivores handle different estrogens in different ways?" because plants themselves have a different estrogenic material. So we could use this simple thing to ask a perhaps more profound question.

There are new concepts coming out of these studies which will describe what we know about environmental estrogens. Here is a model of estradiol; you can see there is a good dose response to estradiol. These are all plant estrogens which are made in great abundance in many different plants. Most of these compounds are much weaker than estrogen. You can see by the doses that they give the same full response but in different orders of magnitude. These estrogens have been shown to have estrogenic activity in a variety of different species, in almost all vertebrate classes including humans. These compounds can also function as anti-estrogens, the anti-estrogenic activity being dependent on the estrogenic chemicals tested. Different plant compounds have different effects on the activity of steroidal estrogens like estradiol; you can see they are pretty good anti-estrogens. However, looking at percent of activity of a plant estrogen, such as genestein, these other plant anti-estrogens really don't have any effect, so the compound can be an antagonist of a male human estrogen. These plant

compounds don't antagonize their own estrogens. If you think about a couple hundred million years of evolution, plants have devolved a strategy where they can antagonize your estrogen but not their own. I think there is an implication here, but I don't know what it is exactly.

We are involved in the kakapo recovery plan which pulls together kakapo estrogens and our yeast-based assays. The kakapo is a parrot in New Zealand, there are only 30 left in the world. It is so rare that they have had to put them on the outer islands so other mammals that have invaded New Zealand over the last millennium won't destroy them. These parrots can't fly, they have to climb trees, but they're not quick at climbing trees, which is why there are only 30 left. The biology of these parrots suggest they are put into a reproductively competent state by phytoestrogens or estrogens in their natural diet. So the kakapo recovery plan asked if they could have our yeast system so they could screen the plants around these parrots and find out when there were peak amounts of estrogen. At that point in time, they would put male kakapos next to female kakapos. I think there are only three fertile female kakapos left in the world. This is one way to take technology that works in the lab and answer questions and apply it in the field.

When we started working with these phytochemicals, we wanted to find out why phytochemicals were involved. Why do plants make estrogens and anti-estrogens? There is some epidemiological evidence suggesting that the phytoestrogens in soy, for example, are protective against breast cancer in cultures that have a soy-based diet. Japan has a much lower prevalence of breast cancer. This is being studied in a lot of other places as well. We have reasoned that soybeans didn't evolve estrogenic compounds a couple hundred million years ago to prevent breast cancer in Japanese women. What were the signals that these compounds were probably biologically set out to do? In the literature, we came across studies reporting a symbiosis between legumes and nitrogen fixing rhizonian bacteria and some of these phytochemicals. Some of the rhizonian flavones (that have flavanoid) turn on some genes in rhizonian bacteria involved in forming nodules around the roots of these legumes that are involved in nitrogen fixation. We asked whether some of these environmental agents interrupt or interact with that system. We took some of the compounds that are most potent in stimulating this nodulation gene bacteria from plants and compared it to other compounds that might exist in the environment. The experiment was a pretty simple one using these kinds of in vitro yeast assays. We were able to take one of the natural compounds in plants into the bacteria. We hoped it might turn on a recorder gene and tell us whether or not these nodulation genes have been turned on with these compounds. We did these studies with natural kinds of stimulators. It turns out that gene products actually have some similarity to estrogen reception. Some other compounds in the family also can stimulate the activity of this nodulation gene in bacteria, and other compounds can actually inhibit them when they are given at the same time as natural factors like globulin. A drop occurs by adding more and more of the different compounds; using fairly high doses of micro-concentrations. One test we did was to take an alfalfa sprout that actually squirts out globulin and other compounds, including a field of bacteria, that has this reporter gene in it that will turn blue. You can see that the alfalfa sprout is pumping out its signaling molecule turning on the nodulation gene for the root nodules for nitrogen fixation. That is why you see blue. If you put these other environmental chemicals in at the same time, they actually physiologically block interaction; therefore, we think we have blocked nodulation. This has implications we are just starting to think about. It gives you another good example of what we're calling environmental signaling and signaling between species.

We're currently collaborating with a group from the University of Hawaii to look at the signals between seaweed and coral reefs. The whole reef makes estradiol in an almost cyclical manner. It gives a peak mid-month; it's like a coral vegetable cycle and literally makes estradiol-17 beta. Those signals - according to the people working in this area - go to seaweed then seaweed signals back to coral. So we are again using our cell systems to study this. It has been very easy to put two graduates students on this project because I have drawn a simulation for the Great Barrier Reef to test our hypothesis.

Here is a list of substances from the literature that I call environmental hormones or signals. It lists a group of synthetic environmental estrogens and synthetic environmental anti-estrogens. You can see that there are a variety different kinds of chemicals with different structures. There are natural estrogens and natural anti-estrogens that function as environmental signals. It may be that these synthetic chemicals are really mimicking the plant estrogens more than they are mimicking the mammalian estrogens. That is a route of discovery that might give us some insight. There are no, as far as I know, environmental progesterones. There are precursors to

progesterone in yucca plants but there are no known effective environmental progesterones. There are some synthetic anti-progesterones, but there hasn't been much work in this area. To my knowledge, there are no environmental androgens but there are some synthetic anti-androgens. There is one published report of a synthetic retinoid (rethocreme), but as far as we know, no synthetic anti-retinoid. As we look at these things, we may find a variety of chemicals that have the ability to block or mimic in a variety of different ways.

There have been reports in the literature for a decade saying that compounds in the environment have estrogen-like effects and are associated with feminization of a variety of species- particularly those living in the water. Some of these have resulted from field studies, others from laboratory studies, and still others from studies which cross over from field to laboratory. There was a very elegant study done by the U.S. Geological Survey where they mapped effects on endocrine systems of fish and correlated chemicals crossing states. This is one area where a lot of these effects are apparently developing. Both males and females are affected. There is a sense that this is going to alter the development of biology in these systems and it is going to change differentiation of key systems. I am going to present some ideas about a mechanism whereby hormones or hormonally active chemicals may act differently toward development. There is a lot of different molecular biological research where this would be the case. In one example, if you give a mouse a variety of different estrogens early in life, when the genital track is developing, you can induce cancer of the vagina or uterus later in life. You have these persistent differentiation defects. DES given to pregnant women is associated with cancer of the vagina in some very low percentage of human female offspring. If however, you give a mouse all of the estrogen you want for as long as you want after puberty, there are no reports of cancer of the vagina or uterus after that treatment. There are apparently critical periods when these cells are programmed to have differentiation effects that lead to cancer as well as a variety of other dysfunctions. We also know that the same kind of exposure to DES will include estrogen response to genes. Our lab and other labs have shown that these genes are upregulated after a critical period of exposure. Lactoferin is the major estrogen reaching the gene in the mouse uterus. It stays on after neonatal treatment with DES as if it always sees estrogen. This is an exquisitely estrogenic responsive gene. There is a 200 fold increase in the message after estrogen. Take estrogen away, it drops down to undetectable levels, give estrogen again, it goes back up. If you reverse the role of effect, the changes that occur through development are associated more with either gene cell organ response. In many cases, you end up with an irreversible change, so if you give DES and other estrogens at a critical period of development of the uterus, lactoferin gets turned on and stays turned on. That cell is going to think it is seeing estrogen. How do these reversible signals become irreversible and what does this tell us about some of these compounds in the environment that may be working at very low levels?

What might be some of the mechanisms that they can use? Another way to ask this question, in terms of the carcinogenesis idea is how can epigenetic changes become genetic? We have been searching for years to see if environmental hormones cause dramatic mutations and have found no evidence of that. The approach we took was to look at a molecular step known to be associated with differentiation, that being methylation of the base of DNA, which is known to occur in the pattern changes during differentiation. These changes in methylation alter gene expression. If you change the methylation of certain plants you actually can get all flower or all stem. The way we think it works is there is a system of either hypomethylation or hypermethylation. When this leads to cancer in rats you see demethylated or methyl groups methylated to CpG sites. You can actually increase gene expression cell growth and that can lead to cancer. Another mechanism is a tumor suppressor gene. If you hyperventilate it -put more methyl groups on - most of the time you get increased gene expression. When you take away methyl groups from these CpG sites you turn on gene expression if you put them in methylation during development. The gene expression is actually shut down. This is a single application but it is the general principle. Our question was can chemicals in the environment alter gene structure and function? Not by mutation. We looked for this for years as have many other labs, and we haven't seen it. Another way to get a structural change that would be permanent to many different cell lines could be through the process of evolution.

Back to lactoferin. We are now doing human studies in parallel with mouse studies. We found that if we put lactoferin into the yeast reporter construct and estrogen response element, it has the promoter site of this gene both in the human and the mouse. Both also had estrogen response element overlapping some other things. If you look in the mouse lactoferin promoter, upstream from where the estrogen

response element is, you see that the estrogen receptor sits down and turns this gene on. Theoretically, there are 5 CpG sites where methylation occurs. So our question was, is the pattern of methylation changed, and is it changed in a time dependent way? If DES is used the first five days of life in a mouse, virtually 100% of those mice will have transplantable epidermal cancers of the uterus. They also will overexpress lactoferin as if they are seeing estrogen, even when there is no estrogen. If DES is used after puberty, on day 30, cancer of the uterus never occurs. We don't get epidermal cancer of the uterus. We don't get a change in the expression of lactoferin - any change there is invariably reversible. The question is: are we changing methylation?

We did a study looking at methylation patterns at different ages of mice treated neonatally with DES. We found a persistent difference in the degree of demethylation depending on the CpG site. When we treated mice after puberty, we did not see any effects across CpG sites. So at a period when estrogen causes reversal effects, the changing of methylation of a single phase and a P450 enzyme can switch its expression from male to female. One methylation on one base can actually change the expression of the gene. This is an exquisitely sensitive. When you give DES to an adult, it shows no effect.

This says that xenobiotic chemicals, in this case the potency in the amount of estrogen, can alter gene structure and function for the process of methylation, which persists and can change the expression throughout life. This gives us some insight into looking at how these signals may be causing some long lasting effects, at least at the molecular level. We have looked at this in the human uterus, with uterine adenocarcinomas. The methylation pattern of lactoferin promoter in human uterine cancer is different than in the normal human uterus at distinct stages of estrogen stimulation.

Let's come back to the whole idea of hormones, genes and development. With the hormonally active compounds, we are right at the borderline in physiology and pathology. These two things are related to the critical period of development; it also takes time for these changes to express themselves. They don't often express themselves as acute abnormalities, but over time there are functional changes that could be inconsistent with normal development or normal intellectual function. The compounds I call estrogens all share a hydroxyl group, or most of them do. Depending on the timing, you can get genetic imprinting if you look at an undifferentiated cell receptor at a level for which we now know there are different estrogen receptors. They can give you cancer cells or infertile cells that respond abnormally. If, in fact this is a differentiated phenotype that is getting these different environmental cues, you have competent cells that are hormonally responsive, so again, you have a way to look at reversible or irreversible change.

Effects of low levels of environmental estrogenic chemicals on the development of the reproductive system

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I want to talk about a unique type of approach my colleagues and I at the University of Missouri have been working on for a number of years. As our research on the effects of hormones in ecosystems during development moved from endocrinology into the field of toxicology, I became intrigued by the essential disconnect that existed between the literature on screening for chemicals, the literature on exposure, and the way laboratory toxicologists test chemicals. It appeared as if none of the information from one domain was having any impact on the way individuals working in other areas were organizing their thoughts and their experiments.

Over the last couple of years, we have developed strategies that I think will be relevant to the EDSTAC (Endocrine Disruptor Screening and Testing Advisory Committee) process. One of the potential impacts of this process (in which screening and testing systems for endocrine disruptors will be developed) is the opportunity to apply this information in some very unique ways. We are collaborating on a project with the National Center

for Toxicological Research and with the VA Lab in Jefferson, Arkansas to develop what is being called an "estrogen knowledge base". You start out at the structural level and move through molecular events, which is critical to understanding how endocrine disrupting events at the mechanistic level occur. Are these plausible ideas and are there mechanistic explanations? Our lab will link that information from in vitro systems into ways to test animals. From that point, you can talk about the intra-animal signals that govern our social interactions; this is something that we have also been working on. We have an article coming out in the next issue of the *Journal of Toxicology and Industrial Health*, which will be an issue devoted to endocrine disruption. Our article will focus primarily on brain development.

I want to talk about linking together various pieces of information; this is going on as a focus between the National Toxicology Program and our program at Missouri. We are very intimately involved in developing these strategies; there is a formal process where we are trying to bring all of this disparate information together. I will present one piece of how we have done this and leave you with one of the surprising outcomes, which was totally unpredicted by current strategies used to test environmental chemicals. These outcomes are raising serious questions about estimates of safety of environmental chemicals based on current testing strategies. This is an unexpected outcome of what we thought would be a rather focused program.

I am going to talk about environmental estrogens, not because they are the most important of the endocrine disruptive chemicals, but because we know the most about estrogen biology. The in vitro systems working with estrogens are more developed than they are for most other systems; we have a 50-year literature base to fall back on in terms of understanding information about estrogen. This huge literature base allows us to use estrogens as an example of what may be applicable to events going on with other types of endocrine disruptors. I am going to talk about estradiol, which is our natural diffuse estrogen. John McLachlan has already told you a lot about synthetic diethylstilbestrol (DES). I am also going to talk about bisphenol isomers. For example, bisphenol A is an estrogenic chemical used as the building block of polycarbonate plastic, a hard clear plastic that is used in many products. It is the building block of resins used to line over 100 billion canned products, it's a protective casing to metal and it is used as a dental sealant. It is one of the 50 top produced chemicals in the world; a multibillion dollar industry.

Another example is octylphenol, which is used in detergents, creams and many other household products. Researchers have looked at the cellular mechanisms of these chemicals and the way their steroid, or a steroid-like environmental chemical acts. If it gets into a cell and binds to a receptor, things happen. Our laboratory assay system detects the presence of these chemicals by taking cells and putting them into a dish of MCF7 human breast cancer cells. As you add estrogen, they begin to proliferate. They don't proliferate in the absence of estrogen, so you can see how much of a chemical is needed to bind to one proportion of receptors. You can then see how potent that particular hormone is in terms of stimulating a growth response or a proliferation response. You can do that with other chemicals and compare the concentrations of these chemicals required relative to estradiol that caused the cell to proliferate. This is a measure of the intrinsic activity of a chemical.

I am focusing on this concept of intrinsic activity because there are lots of things that have to happen for a chemical or a hormone to get into a cell, where its intrinsic activity will be realized. As an example, after ingesting a chemical, it is subjected to a highly acidic environment in the adult stomach. This would not be true in a newborn where you have a very different type of gut environment; in the adult there are very well developed bacterial colonies that you don't necessarily have in a newborn. There are different groups of exposure, and it is important to assess the front-end events that determine how much of the chemical is actually going to get into the blood. It passes through the liver and the maturity of the enzyme systems in the liver will change dramatically throughout early development. This is going to influence the way an organism responds. All of this comes to critical phases. Is something happening in the adult ever going to be predictive of what is going to happen in a newborn or during fetal development?

Finally, these chemicals start partitioning in different parts of the body. Some of them are sequestered in fat. Women mobilize fat when they are pregnant. Where does it go? Into the fetus or into their breast milk. So, sequestering persistent organic pollutants can be a serious problem depending on when in life you start to mobilize these sequesters. I am going to focus on the system that exists in the blood that carries these relatively hydrophobic molecules, little lipid-like molecules in the blood. Plasma proteins and other systems that exist in the blood transport these chemicals and control their biologically active levels. The front-end events determine how much of a chemical gets into a cell and its intrinsic activities tell you what it will do once it gets in there.

Up until now, the focus has been on what is

going on at dose at target at the receptor. Blood is very important in terms of altering the response profile. When you put these chemicals in blood very different things happen in terms of the chemical getting into cells. This is the type of thing we have been doing. A standard MCF cell and cell culture assay system starts out with a number of MCF7 cells, but no hormone. You add increasing doses of estradiol, and by the end of the fourth day, there is an increase in the number of MCF7 cells. Fifty percent of the maximum number of cells is used as the reference point for comparing chemicals in your dose that is effective in inducing a 50% response. Chemicals such as coumestrol take a dose about a thousand times higher to cause MCF7 cells to start growing relative to estradiol. If you divide the dose of coumestrol (for 50% response) into the dose of estradiol (for 50% response) you get a relative potency. This is an estimate of its intrinsic activity, that is, after it gets into the cell and binds to the receptor. These studies are conducted in the absence of factors in blood that would modify any of those events. So we're talking about the amount of this chemical in the medium (which is the pre-dose at target) that is actually getting into the cell because there is nothing stopping that from happening.

Let's focus on how sensitive this system is. We are seeing proliferation of MCF7 cells at way below 1/10th of 1 trillionth of a gram of estradiol in a milliliter of medium. When you think of environmental estrogens as being weak, that is what they are weak relative to. If something is 1000 times less potent than that, it is still working in the part per trillion range. We constantly say, "Oh this is present in the part per millions - isn't that such a small amount?" When you look at the data from a cell proliferation study, you see something very interesting about the way estrogen operates in MCF7 cells. That is, it takes less than one trillionth of a gram per milliliter of medium. Here we are down to 0.27 occupying 1% of the total number of receptors available in that cell to give a 50% of the maximum growth response that is going to be induced in that situation. Detection occurs at a log lower dose than that. That is, the cell is responding at way below 1/10th of a trillionth of a gram of estradiol per milliliter of blood medium. The physiological range of estradiol is in this 1% to 5% to 10% receptor occupancy rate. In the rat, for instance, diestrus levels of estradiol are about 0.27 and proestrus levels are in the 2 parts per trillion range; this is the physiological range of activity. This has been an enigma for endocrinologists for years - that so few receptors have to be occupied in order to see this huge response. It tells us that the system is highly tuned for detection, it is incredibly sensitive. A detectable change in response can take place with only a handful of receptors being occupied, which results in an extremely small number of receptors changing in occupancy.

We did the same experiment with bisphenol A. The American Can Institute estimates that a person who eats an average amount of canned food is getting 6 micrograms per day of bisphenol A. That is chemical industry data. The data from Ana Soto's article showed that during the first hour after having dental sealants put on, you can get up in the 900 microgram range of exposure. So depending on the kind of product and what is going on, you are getting significant hits of bisphenol A. Our data on bisphenol A for MCF7 cells shows that it takes about a nanogram per milliliter of medium to occupy 1% of receptors to get a response. The biologically active range of this chemical is in the 1 to 10 nanogram per milliliter range or 1-10 parts per billion.

One of the major questions we face in this emerging set of disciplines is how could we get effects at such low doses of chemicals? To an endocrinologist, it makes intuitive sense that if a chemical gets into a cell and it can reach the receptor, it should cause a response; it is biologically active. But in toxicology, where you are working with much higher doses, a part per million is an incredibly small amount. So this new approach is very different than the tradition of toxicology, particularly looking at acute toxicity. What was known about bisphenol A was based on a study where 1 part per 1000 and 0.1 part per 1000 was the lowest dose from which they calculated a no effect dose and developed safety data. One interesting thing about this is you have already totally re-saturated the receptor system. That dose response curve of moving from 1 part per 1000 to 0.1 part per 1000, you are moving from 100% receptor occupancy to 100% receptor occupancy! It is hard for me, as an endocrinologist, to imagine how that could lead to a change in response; you are supposed to change the receptor to occupancy to see a change in a hormonally mediated response where the mediating event is binding to that receptor. So we decided to actually investigate toxic effects versus hormonal effects to see if there was any relationship between them.

We took MCF7 cells, put them in the dish, and then added increasing amounts of estradiol. At the 10^{-13} range, below one part per trillion, you are beginning to stimulate the MCF7 cells to start proliferating. They proliferate for a fairly wide range of doses. You get maximal proliferation then you get into the part per billion range, where the amount of proliferation starts coming down. When you get into the high part per billion range you kill the cells; that's the toxic dose.

Interestingly, the same dose of bisphenol A and estradiol kills MCF7 cells. That event has no predictability of how hormonally potent those chemicals are. In fact, what you see in this assay is that bisphenol A is about 10,000 times less potent that estradiol. It's active in the low nanogram per milliliter range and estradiol is potent below one part per trillion. It's about 10,000 times different. They kill cells at the same amount, but this killing effect is not predicting the hormonal activity, it has nothing to do with hormone binding. These cells are going through a transformation. Unfortunately, when this occurs in people, they no longer have estrogen receptors. If you put them in the right conditions, they grow like crazy with no estrogen. That transformation is what kills you with breast cancer because you can control cells at the top but not at the bottom. These are transformed cells that have no estrogen receptors. We keep adding higher and higher doses of estrogen, estradiol and bisphenol A, and eventually, the same dose that killed the cells with estrogen receptors kills the cells without estrogen receptors. It is not a estrogen receptor problem, it's a toxic event. The conclusion I have reached is that acute toxicity, studied in high dose toxicological custom experiments, tells you nothing about how a chemical reacts as an endocrine disruptor in terms of operating through receptor-mediated mechanisms. I think the data pretty much tell the story.

So we are studying a teratological event with very high doses of chemicals; we need to know this information. I am in no way saying I think it is not important to test high dose effects. I am saying that they are not going to predict endocrine disrupting events; this is not an endocrine disruptive kind of outcome. There are much more subtle functional kinds of things we have to think about other than gross teratology. John McLachlan was talking about this cell differentiation event where we start out life as a cell and two cells end up different because there are very subtle differences in the amount of hormones. We are not talking about the presence or absence of hormones. Males have just a few more parts per billion testosterone than females and this leads to a totally different event; incredibly small differences in concentration changes and timing. There are critical windows of time during the differentiation event when cells are irreversibly imprinted with how they will function. In order to get to the cell, these chemicals have to go through the blood. There was no blood in the cell assay systems I have been describing. All measures of potency were based on the assumption that the chemical went through our mouths and into the cell which, of course, doesn't happen.

So we begin to focus on events. For example, what is happening to these chemicals in the blood, especially during pregnancy and fetal development, which is a much more critical issue than in an adult? During pregnancy, there is an increasing number of binding events going on in the blood. The idea that during pregnancy we are exposed to so much estrogen is really an illusion. The biologically active fraction of estrogen is pretty much the same in a pregnant woman, a fetus, or an adult. While the total amount of estrogen that is measured during pregnancy is high, it is not necessarily biologically active; only the unbound portion can actually dialyze into the cell. There's a lot of confusion over this very important piece of information. We did work that showed levels of estradiol in rat fetuses are very high. The total amount of hormone (the free biologically active amount of estradiol throughout the last four days of pregnancy and then on into adulthood) is exquisitely regulated by plasma binding proteins produced by the liver in the fetus. You will notice that in the rodent, the level of free estradiol is at a 0.2 or 0.3 part per trillion range, exactly the amount of estradiol that produces proliferation in human cells. We have every reason to believe that the human system and the rodent system are equally sensitive to estrogenic events.

What about xenobiotics? For decades it has been known that a wide variety of xenobiotic chemicals - estrogenic chemicals - don't interact with these binding proteins the way estradiol does. Our laboratory wasn't the first to show this, we are just applying this to the issue of how potent these chemicals might be in living systems. This is absolutely critical. A chemical might be 1,000 times less potent than estradiol in an in vitro system with no blood present. In the presence of fetal blood, however, 999 of those estradiol molecules may get blocked, but if one gets into the cell then all 1,000 molecules of the xenobiotic gets in. Your estimate of this being a very weak chemical suddenly is out the window. This is critical in the fetus with so much estrogen, which normally is not getting in. Some xenobiotics can bypass this critical barrier system that protects the fetus from excessive estrogen. Data from studies conducted in the 60's shows chemicals such as DES and bisphenol A are not being blocked in the same way estradiol is by these plasma binding proteins. What are the consequences of this, and how do you go about studying this?

We have incubated MCF7 cells in the presence of estradiol whose receptors are filled with radioactive markers. You add a chemical which, if it is an estrogen, will bind to the receptor and competitively displace the radioactivity. A curve results; no chemical present gives 100%

radioactivity. As you start increasing the dose of this estrogenic chemical, the amount of radioactivity in your system begins to decrease, eventually reaching zero. You can do this with estradiol or any other xenobiotic estrogen, such as octylphenol or bisphenol A. Again you use the 50% displacement point to make your relative potency comparison. Octylphenol is about 1,000 times less potent than estradiol, and bisphenol A takes a higher dose to displace the radioactivity by binding to the estrogen receptor. The parallelism of these curves demonstrates there is binding to the estrogen receptor that it is specifically displacing the bound radioactive estradiol. These are estrogenic chemicals that are being detected; we can also get an idea of their potency.

We took human blood, put it into these cells and ran the experiment again. We found something very interesting - the potency of octylphenol and bisphenol A in relation to estradiol changed places in the presence of human blood. In the presence of adult blood, bisphenol A looks to be a more potent estrogen than octylphenol. Adult human blood has very little binding protein compared to fetal blood. In the presence of fetal blood, bisphenol A and estradiol begin moving very close together; it looks as if octylphenol is being inhibited by these proteins to a much greater degree that estradiol. In this situation we have two environmental estrogens: bisphenol A is bypassing the barrier system and octylphenol is blocked by the barrier system to a greater degree than we had thought possible. The predictability of in vivo potency can really be determined by this kind of additional information, which we can use to predict the dose of bisphenol A that would be biologically active relative to estradiol.

We then decided to use the development of the prostate and the testes as endpoint markers that we know are very sensitive to the presence of estrogen. The first task was to determine the dose of estradiol - our reference molecule - that would alter prostate development. We already know a lot about both the free level of hormone in the blood and the level of receptor occupancy that needs to be increased in order to detect the difference. We were interested in benign prostatic hyperplasia. We implanted capsules of estradiol in pregnant mice and increased the amount of estradiol in the blood of these fetuses by 0.1 part per trillion. One day after the initial development of the prostate, we dissected out the urogenital sinus and scanned it into a computer which reconstructs the entire organ. After 24 hours of development in an animal given 0.1 part per trillion estradiol in a milliliter of that fetus' blood, it dramatically induced biogenesis; it was detectable on the first day of development in this organ. The rat starts out life with an abnormally enlarged prostrate. In adulthood it ends up with more cells in the prostate or androgen receptors per cell. A little bit of estrogen has upregulated androgen receptors 6 fold, a 25% increase in estradiol caused a 300% increase in androgen receptors, and a 50% increase in estradiol caused a 600% increase in androgen receptors.

This is classical synergism. Estradiol synergizes with hormones such as oxytocin or progesterone to give you a much greater outcome than you would get with these hormones alone. This is a perfect example of synergism as a potential outcome of exposure to these endocrine disruptors. We gave 0.1 part per trillion of estradiol to increase prostate size. Our prediction was that 2-20 parts per billion of bisphenol A would be biologically active but that octylphenol, because the proteins were blocking it, would not be biologically active at those doses. The offspring of the mothers fed for 7 days of pregnancy at 2-20 parts per billion bisphenol A, is well within the range of what people are getting out of products they are using with this chemical in it. We call this an environmentally relevant dose. Our physiologically based mechanistic studies have told us this should happen.

What we have here is a complete paradigm shift. You begin this process by determining the concentrations of estrogen that would normally alter development, then you get an equivalent dose of an endocrine disruptor and examine it in an in vivo system. We are using in vitro mechanistic information to develop a biologically-based physiologically rational base for dosing animals. Instead of using assay systems to detect whether a substance is or is not an estrogen, we now have a way to dose animals within a hormonally relevant range based on the outcome of an in vitro experiment. You can then test the complete dose response rate. We haven't done that with these chemicals yet, but NIH has done it for DES, and we've seen the same outcome for estradiol and prostate size. We feed pregnant female mice (during the last 7 days of pregnancy) doses of DES starting at 2 parts per trillion and moving up to 200 parts per billion. Nothing else was done. What you see is a very similar result. Toxicological studies start at this very high dose- that is maximum power rated dose that causes some adverse outcome in the internal organs. You move up to the line that would be the no effect dose. Why hasn't this kind of thing typically been seen in a toxicological study?

There is a paper coming out shortly in the *International Journal of Toxicology* that surveyed the toxicological literature. It is estimated that of the 500,000 papers in toxicology published during this century, less than 1,000 have tested chemicals using a paradigm like this one, where we can go below the

NOEL that could possibly have shown this outcome. Why isn't this commonly seen? Because nobody is designing experiments where you could see it. In the 1,000 papers in which it was reported, hundreds have seen low dose effects below NOEL effects. If you look for it, you can find it.

John McLachlan's lab has done radioactive injections of DES which provided us with information about how much gets through the mother, across the placenta, into the baby and directly into the reproductive tract. We knew this dosing regime was based on that kind of information. If you have transport information about metabolism and also know the intrinsic activity of an estrogen, you can develop an absolutely biologically based dosing regime that is not based on the assumption of what an acute toxic dose does. This is a red flag in terms of current testing strategies by EPA. The maximum tolerated dose had at most one mark below what is the standard toxicological testing procedure. This shows the testing procedure started at a maximum tolerated dose that causes 10% decrease in body weight. A few doses up in this high dose range are given and then you extrapolate down. As Gina Solomon was saying yesterday, doses below the threshold are considered safe because the system is off, up and to the point at which you reach that threshold. What do we know about this whole process?

First of all, this system is not a linear performance. Endocrinologists don't think in terms of linearity except in an extremely low dose, maybe in the 1 to 5 to 10% receptor occupancy range. Beyond that the system becomes explicitly non linear and gives you diverse types of functions similar to ones I have shown. This is true for both in vitro and in vivo studies. Using bisphenol A and referencing the NOEL of a high dose, we were getting effects 25,000 times below the published NOEL. We have similar data for methoxychlor, which is currently used in insecticides, and data for DDT. The NOEL is not predicting, in our laboratory, outcomes that I clearly consider adverse. We are changing behavior, we are making the prostate hyperplastic, hyper-responsive to hormone risk factors for prostate cancer. I consider these adverse events.

The assumption in toxicology is that the control is at zero dose. But there is a huge body of literature showing that if you block the synthesis of estrogen in rodents, if you give receptor antagonists, you alter development and the amount of estrogen in the blood is sufficient to occupy the number of receptors necessary to stimulate responses. All this leads to the conclusion that the amount of estrogen in the blood is already above threshold. If it is already above threshold, what is the external dose required to reach that threshold? In an untreated population, there is an irreversible effect due to an indigenous chemical, the threshold dose for this chemical has been exceeded and any exposure dose will not display thresholds. It's theoretically impossible. If this is true for estrogen, then it has to be true for the androgen response system. We can't have males without androgen working. Progesterones, glucocorticoids and all of these other systems are active. If you interfere with these systems, there can't be a threshold of events, yet the concept of safe dose in a threshold is the whole basis of regulating different toxins.

We include in this paradigm inversion a no-threshold assumption that there can be inverted 'U' functions. Not that all functions are going to be inverted, they don't need to be. However, the possibility has to be taken into account in dealing with these chemicals. If there are inverted functions, then the NOEL safety factor approach simply doesn't work. What we need to extrapolate from all this is the fact we need to test for low doses. This is true for estrogenic chemicals, and it could very well be true with many other chemicals. This use of an environmentally relevant dose, a physiologically based mechanistic approach, is possible where you have a screen system that tells you about the potency of a chemical and how to work with it in vivo. We are not going to have all chemicals showing up positive in our assays. Does that mean we should assume they are not a problem if they don't show up in estrogens or anti-androgens? The answer is no. One way around this is to not only attack the acute toxic dose, but to look into the exposure literature and link that into the testing paradigm. Find out what populations are being exposed to and use that information to test these lines. What I want to know is what are these chemicals in our body doing? I have seen from my own data that testing doesn't tell me anything about that. That is a real concern. We should explicitly test low doses that are environmentally relevant and are based on exposures.

The next concern is the issue of mixtures; what about potential synergy, types of interaction, etc.? For example, what are the anti-hormone effects of hormone 'X' playing against each other? We must try and find out what populations are being exposed to in terms of mixtures. We can't test every chemical against every chemical but we can create environmentally relevant mixtures that are appropriate for specific populations and test those. Of course, industry should tell us what is in their

products so we know what to look for. It's a very serious problem if you don't know specific uses of these products and what is in them. We also need to monitor during pregnancy. The only way we are going to know if there are fetal effects is to look during pregnancy and then do prospective studies following those cohorts. You can test for DDT in a woman dying of breast cancer and not find a relationship with breast cancer. Let's say, there is no effect of endocrine disruption in breast cancer, when we are proposing it's an embryonic imprint that occurred 60 years before and those chemicals were no longer there nor detectable to give you an effect. You have to ask the right questions. Then comes the idea that there are critical life stages that have to be looked at. In the EDSTAC process, where you are coming up with screening blood, I think we are going to be very disappointed if all screening is done in adults. I can't believe there is anybody here, after hearing the talks yesterday and today, that thinks an adult is going to be the marker of these things causing damage to our development of babies and children. Again, we have to look beyond the gross teratology.

Using fish to monitor for reproductive endocrine disrupting compounds in environmental waters

Erin Snyder, Michigan State University

Ms. Snyder is a Ph.D. student in zoology and environmental toxicology at Michigan State University, where she is also a research assistant to Dr. John Giesy at the Aquatic Toxicology Laboratory. Her research interests are primarily in the area of biochemical, cellular and physiological effects of contaminants on animals, particularly the effects of endocrine modulating compounds on reproduction and development. Ms. Snyder received a B.S. degree in biology from Thiel College.

My work looks for estrogen in the environment that may be coming through wastewater treatment plants. What precipitated our interest was research in the United Kingdom that found intersex fish near wastewater treatment plants. It was the first noticed by anglers. Intersex fish are fish that have both male and female reproductive structures. That does occur naturally in some fish species but not in the ones we are studying near wastewater treatment plants. Researchers from John Sumpter's laboratory in the United Kingdom caged rainbow trout outside wastewater treatment plants in rivers that were impacted by the effluent. They found some endpoints that were indicative of reproductive endocrine modulation. One of these endpoints was vitellogenin induction in male fish. Vitellogenin is a female specific protein that is not normally found in male fish. We also found some induction of vitellogenin in immature fish, which is also not normally produced. I am following up on another study that took place in the United States by studying sex steroids that play an important role in fish development and reproduction. Sex steroids are involved in developing the reproductive structure and sexual differentiation in young fish. They are involved in seasonal development in preparation for spawning. Fish only go through these cycles once a year. They also are involved in sexual behavior, which leads to spawning and induction of vitellogenin for egg reproduction.

Sex steroid synthesis in the female occurs in the ovaries and interrenals. The primary sex steroids in females are estrogens - including estrone, estradiol, and estriol - and testosterone. In males, the major organs of sex steroid synthesis are the testes and the interrenals, which produce estrogen, testosterone and 11-ketotestosterone. This is a representation of the hypothalamic-pituitary-gonadal axis. The hypothalamus produces a gonadotropin releasing hormone (GnRH) which stimulates the pituitary, producing gonadotropin I and II which are analogous to FSH (follicle-stimulating hormone) and LH (luteinizing hormone) in mammals. These stimulate the gonads to produce sex steroids. Estrogens can feed back to the gonads to upregulate their own production, or if they can feedback negatively to all three (the hypothalamus, pituitary and gonads) at different stages of sexual development. An impact at any one of these points in the axis could have serious effects on the sex steroid production. This is a very complex pathway; major interference at any point can seriously impact the production of testosterone, or 17-beta estradiol, which is what I am interested in. In our lab we use MCF7 blue which contains a firefly gene so when estradiol applies to DNA it produces light, and we can measure light production.

When working with fish you must take into account the natural variation in the levels of circulating sex positive steroid. There are greater

levels of circulating sex steroids in gonadotropin hormone (GtH) during reproductive seasons; there are also variations in sex steroid and GtH levels over spawning cycles within the reproductive season. Some fish, gold fish included, will spawn several times within a reproductive season. Other fish, like carp, will only spawn once. There can be considerable variation during a day. Diel cycles of sex steroids and GtH for some teleosts can vary widely in a twenty-four hour period. Stress, including the simple stress of handling a fish or confining it, can reduce levels of sex steroids within hours, which is important to keep in mind when you are sampling fish. There are many sources of estrogen in the environment that you must be aware of. When evaluating the effects of these compounds on fish, you should keep in mind that they are exposed to many hormones from different sources. I don't believe that estrogens are the only sex hormones in the environment that could be causing effects: we should also be looking for androgens and progesterone.

In vivo biomarkers that can be used to look at reproductive endocrine disruption in teleost fish include altered gonadosomatic index, which is the ratio of the size of the mass of the gonads to the mass of the gutted carcass. That is indicative of seasonal reproductivity. Another method is the altered sex steroid profile, which is a measurement of estradiol to testosterone ratio, or the estrogen to androgen ratio. That is important because even if testosterone is considered to be a male hormone, a female gold fish can have higher levels of testosterone. In female fish, the estradiol to testosterone ratio is greater than one and in males it tends to be less than one. Absolute levels may not give you a whole story. Other in vivo biomarkers are vitellogenin induction, histologic changes in the gonads, and altered secondary sex characteristics. The level of testosterone in particular has been shown to be important in expression in secondary sex characteristics. Other in vivo biomarkers include gross abnormalities in the reproductive tract and altered sexual behavior.

Vitellogenin in fish is one endpoint that has lately received a lot of attention. Vitellogenin, which is a glycolipophosphoprotein, is a precursor synthesized by the liver in response to estradiol. While there are other factors that can also affect it, it's generally accepted that if you find above normal vitellogenin induction in fish, it is due to estrogen. It's carried in plasma and sequestered in developing oocysts, where it is cleaved to lipvitellin and phosvitin, which are the principle reserves of eggs. Vitellogenin is a very complex high molecular weight plasma protein. It's a useful biomarker because male and female fish both have the genetic machinery to produce it. Immature fish normally don't produce it because they usually don't have levels of estradiol which are high enough to induce production. It is inducible in both male and immature fish and in female fish that aren't in their proper reproductive cycle by exposure to estrogen agonists, both by water and by injection of estrogen.

This is a summary of several studies evaluating reproductive endocrine functions in fish affected by sewage treatment plant effluent. A study in the United Kingdom on rainbow trout found vitellogenin induction in male fish, increased gonadosomatic index and polysomatic index in the male and in sexually immature fish with vitellogenin induction. Studies conducted in Minnesota and in Lake Mead, Nevada, on feral carp found vitellogenin increase in sexually immature fish. This phenomenon is not confined to fresh water fish. There's a marine study with flounder where they found vitellogenin induction along with testicular abnormalities and an increased gonadosomatic index. Originally it was believed these effects might be due to alkylphenol ethoxylates or their degradation products. It turned out that the binding of these compounds to the estrogen receptor is rather weak except in places where there is gross contamination in the environment. It didn't make sense that the level of these compounds in the environment could be responsible for the level of response they were seeing. They thought it might be a kind ethinyl estradiol which is a component of oral birth control medication. Sumpter's lab presented research on ethinyl estradiol in wastewater at a recent meeting in Washington DC. They thought it might actually be present in concentrations high enough to account for the effects that were being observed. Ethinyl estradiol is very similar in structure to 17-beta estradiol but it is synthesized to be more stable which gives it a longer residence time in the body. It makes sense that it might be coming through in wastewater treatment plants.

Why use goldfish, when there are more sensitive species such as rainbow trout? We wanted to work in Michigan, and the rivers I was looking at don't normally support rainbow trout. I want fish that are happy and alive, so I work with goldfish, which are also commercially available. There are several advantages to working with goldfish. There is an adequate volume of blood for measurement of reproductive steroid hormones, so you can do repeat blood samples. They are easily cultured and induced

to spawn in the laboratory. If you've done work with fish, you know that's not an easy thing to do. Goldfish organs are large and distinct. They can tolerate the range of water quality parameters encountered in wastewater effluent streams. A rainbow trout or a sensitive species cannot survive under these conditions. In addition, there are a lot of endocrinology data available on gold fish. If I see an effect, I can try and figure out what the mechanism is rather than going back and doing basic research to figure out what is going on. There are several disadvantages to using goldfish, as well. They lack a number of obvious secondary characteristics. They may require feeding when used in field cage studies, and they can differ significantly from the native species in their sensitivity to xenobiotics. After being raised for generations in the laboratory, they are tolerant of a lot of things that other species in the environment might not be. In addition, commercially obtained fish can have a heavy parasite load; you may have to treat them with all kinds of chemicals to get rid of parasites, perhaps affecting results. A lot of times fish that have been raised in hatcheries have been treated with hormones. Sex steroids are used in fishery applications to alter the sex ratios in fish. We raise fish in our own laboratory so we know what they have been exposed to. Finally, goldfish are a multiple spawning species, which can complicate the interpretation of your results.

We selected wastewater treatment plant sites in Michigan; most were small, primarily having municipal sewage, although some had industrial input as well. The cages were submerged in the water, and we placed 20 fish per site (10 males and 10 females) and mounted the cages directly in the effluent flow for six weeks. If there was going to be an effect, there was no point going with upstream or downstream caging, we wanted to see the biggest effect possible. The fish were checked for mortality and illness weekly, and we monitored water quality. We looked at the standard length and weight, the gonadosomatic index, the general condition, and we collected blood for vitellogenin plasma, 17-beta estradiol, testosterone and 11-ketoestrogen. We collected samples of the gonads for histopathological examination to look for abnormalities and to determine the sexual stage of the fish, and we looked for in vitro gonadal steroid production.

For in vitro gonadal incubation studies, we removed the gonads from the fish and weighed out specific portions. You want to keep the portion the same from sample to sample, in order to get the follicles in the same stage of development. When you incubate them for a specified amount of time, you assay the hormone in incubation media. You add gonadotropin, precursors, and co-factors to investigate the capacity of the gonad to respond to stimulus following exposure of the fish in vivo to a toxicant. This is a way of separating out the gonadal steroid production versus steroidal production from the adrenals to facilitate the study of the mechanisms of action. For example, say I found a reduced level of sex steroids in a fish at one site but not at another site, and it turns out that the gonadal steroid production is the same at both sites. It could be that there was induction of lipid that increased the carrying ability of sex steroids rather than a direct impact on the site. The resulting measurements correlate well with plasma hormone levels, sex steroid levels and for their use as biomarkers.

There are a number of considerations to keep in mind when using plasma sex steroids as biomarkers. Temperature has a strong effect on plasma sex steroid levels; effluent water temperature changes over time, so you have to make sure that the temperature is pretty close at all sites. A period of rainfall has an effect; rainfall can induce fish, goldfish in particular, into spawning. The presence of appropriate spawning areas and materials can alter a fish's sexual development, and their reproductive readiness for a potential mate can have an influence on this. For example, male goldfish won't come into complete sexual development and reproductive readiness without close proximity to an ovulating female. In addition, stress and handling can reduce sex hormone levels and the stage of gonadal recrudescence.

Finally, there are several other important endpoints that we assess for reproductive toxicants in our studies. We look at mixed function oxidase induction, aromatase activity (aromatase is an enzyme that converts androgen into estrogen), and the hepatosomatic index, which is a measurement of the liver weight divided by total body weight. We also look at the condition of the fish and the weight, evaluate them for stress by looking for stress proteins and plasmas stress hormones, and finally assess population level effects.

The EPA perspective and the development of a consensus for testing

Gary E. Timm

Mr. Timm is senior technical advisor in the Chemical Control Division, Office of Pollution Prevention and Toxics, U.S. Environmental Protection Agency. He is currently working with the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to implement testing requirements of the Food Quality Protection Act of 1996. Within EPA, Mr. Timm has been involved in establishing corporate average fuel economy standards, and serving as co-manager of the Toxic Substance Control Act test rule for testing hazardous air pollutants under the Clean Air Act of 1990. He has an M.S. in organic chemistry from the University of Minnesota and an M.A. from the Humphrey Institute of Public Policy.

A lot of what I was going to cover has been touched on by previous speakers this morning. I do want to briefly discuss the science view of EPA, legislation leading to the EDSTAC (Endocrine Disruptor Screening and Testing Advisory Committee), what's currently going on at EDSTAC, and then finish with some information on EPA and the international research program.

EDSTAC presented and published the science view on endocrine disruptors in February 1997. This is a good overview of the state of the science, but it is unfortunately dated, as research papers were cut off in January of 1996. A lot of new work has obviously gone on since that time. Some of this new work is posted on the EPA web site (www.epa.gov). To get to the EDSTAC link, go through the Office of Pesticides and Toxic Substances page. This special report grew out of an Agency wide colloquium on endocrine disruptors. The endocrine disruptor issue is not a new one; it has been brought into public prominence since the publication of Theo Colburn's book *Our Stolen Future*. The special report provides EPA's only definitive science statement on endocrine disruption at the present time. We are awaiting the National Academy of Science (NAS) report, which won't be available until some time in 1998.

I want to talk about the epidemiological evidence on endocrine disruptors. There's work in the Great Lakes area and in North Carolina looking at neurologic dysfunction in children exposed to PCBs. The most controversial studies are those looking at whether sperm counts are declining. European studies have found declining sperm counts, while U.S. studies have not. Certainly, no one debates the increase in the number of cases of cancer of the prostate, testicles, breast and cervix, as well as endometrioses. However, the data have not been able to correlate that these are, in fact, from endocrine disruption. Two studies not in the 1996 report are most interesting to me; they show for the first time in humans some of the things we have seen in wildlife. In one study, the ratio of children born to mothers who were exposed to dioxin from the 1976 Seveso, Italy, accident was skewed to 60% girls and 40% boys during the first year after the release. Over the next 8 - 10 years, it gradually returned to the normal 1:1 boy/girl ratio. The second study was the contamination of rice oil in Taiwan, where boys born to exposed mothers had diminished penis size. This correlates to what we have seen in several instances in wildlife.

The EPA position is composed of rather gray bureaucratic statements at this time. In light of what has occurred and what has been reported in the press, it is worth saying that EPA is not backing off of its view concerning endocrine disruptors. That view takes the whole area of endocrine disruption in the environment as a serious issue. We recognize the potential of adverse effects when people are exposed to endocrine disrupting chemicals. The wildlife evidence is a lot stronger than the human evidence and there is disagreement regarding the scope of the problem, whether we are talking about hot spots in the environment or about much broader multilevel environmental exposures. As we have heard from Dr. vom Saal, the evidence is growing that we are talking about a much broader exposure. The hot spots are where we discovered there was a potential problem. I believe the Agency has defined endocrine disruption to be a mode or mechanism of action, not necessarily an adverse effect per se. There are lots of potential adverse effects associated with endocrine disruption including carcinogenic effects, developmental effects, and learning behavioral effects. That is EPA's definition. There are a lot of places where things can go wrong, where the actual mechanism can interfere with transport or binding at the receptor. We recognize that endocrine disruption is of concern to vulnerable ecosystems, but that not all species are going to react the same. Developing embryos, as we heard this morning, are at greatest risk.

EPA has committed upwards of 10 million dollars to research on endocrine disruption, and is only one of several agencies that is involved in the research. The EDSTAC is trying to fill the gaps on the test tube side. We have seen and talked about the

evidence that led to public concern, which in turn led to the passage of the Food Quality Protection Act in August 1996. This Act mandates that EPA develop an Endocrine Disruptor Screen and Testing plan by August 1998, and that it implement that program by August 1999. EPA will report back to Congress on the program's progress by 2000. The Act states that, at a minimum, you must test pesticides, active ingredients and inerts for estrogenic effects as they may affect human health. The Safe Drinking Water Act further says that EPA can test any substances found in drinking water to which a substantial number of people may be exposed. The Food Quality Protection Act gave EPA permission to test for endocrine effects and environmental effects for any substance on the toxic inventory as well as water contaminants and pesticides. How did EPA react to this mandate? EPA felt it would be necessary to involve outside experts, as a great deal of the expertise necessary to implement the Act was certainly outside the Agency. Furthermore, this is a contentious issue in science which will undoubtedly result in a lot of controversy. The recommendations and conclusions the Agency reaches will be debated, and any regulatory program that required data generation is subject to litigation. Therefore, EPA involved all the major stakeholders and groups to help develop a testing strategy. There have been workshops that have looked at individual assays, mammalian assays, and environmental screening assays. Anytime a federal agency wants to achieve a consensus recommendation group, is must establish a Federal Advisory Committee, as EPA did in October 1996. The EDSTAC has approximately 45 members representing interests from the chemical and pesticide industry, environmental and public health groups, federal and state agencies and labor. We are trying to have people wearing two hats - a science hat as well as a representational hat.

When the EDSTAC first met in December 1996, the members generally agreed and understood what the law mandated. However, the EDSTAC did not want to restrict the debate to health effects since ecological effects have really been where this phenomenon was shown to be the strongest. Furthermore, confining the debate to estrogen was not adequate; anti-estrogen, androgen and thyroid effects also needed to be looked at. This represented to EDSTAC a credible minimum. While there are many more complex problems, given the time constraint - two years to develop the plan - it was felt that this was something doable. Addressing all 50 or so vertebrate hormones was not feasible.

The EDSTAC also needs to address important mixtures as well as single compounds. The EDSTAC divided its members into a priority setting workgroup and a screening and testing workgroup. The first question involved how to select and prioritize chemicals for screening and testing. They decided there are large macromolecules (polymers) that they could exclude that would not be bio-available. This decision wasn't as easy as it sounds. We initially thought that a cut off at a molecular weight would be a decent definition. However, it turns out that compounds of somewhat larger molecular weight can get through to a developing fetus, as the cell junctures are not as tight in an embryo. That was just one of the subtleties and difficulties the EDSTAC faced. We decided to proceed in a two-tier fashion - a screening tier and testing tier. Thus far the screening and testing work has focused exclusively on trying to define what should go into the screening tier. We've talked about the transcription activation assays and the complexity of the issue, about using the Hershberger Assay in males and the Uterotrophic Assay in females. We're mostly looking at mammalian systems, and one of the questions is "do we need to look across a wider group of taxa than that?" When you get beyond the mammalian assays, it is difficult to make some choices. Other assays are not as well developed, and are not necessarily validated. But we have also been looking at bird assays, fish assays, and a turtle egg assay. Another question is "for the purpose of identifying activity, how well do mammalian assays predict endocrine disruption?" Erin Snyder indicated this morning that there are some hormones that are important in fish that are not specific to mammals. We have those kinds of issues as well.

How do we evaluate the toxicity in mixtures? The EDSTAC is taking a fairly pragmatic approach. We can't conceive taking a large number of compounds and contaminants and doing even binary tests on those things. The strategy is to look at mixtures that are important from a human exposure standpoint, for example, the composition of toxins in breast milk, which is very important in human development. Perhaps we should be looking at a group of pesticides and herbicides that a homeowner would use around the house, or looking at exposure opportunities that might define a reasonable set of chemicals that would be expected to interact. That is the approach we have been discussing. We have not yet faced the low dose effects issues and related implications on testing. We are also discussing possible problems resulting from looking at all the assays together, and applying that to the weight of evidence. There are major uncertainties about how

much of a chemical exposure is necessary to cause adverse effects. We are getting feedback from researchers that in fact it can be very, very low levels. Far lower than we normally think of from a toxicological standpoint. Good exposure data are extremely hard to come by. We are trying to develop tools in addition to monitoring programs to help us better understand some of those things. We are looking at high exposure, isolated effects but now are we talking about a potentially broader problem from low level exposures. Are our current test protocols adequate? I think the answer we heard this morning was "no".

We have a new set of guidelines for reproductive and developmental effects, and we can add more endocrine sensitive endpoints. Reproductive effect studies looked at female cyclicity, male sperm count and morphology. I think we are going to have to rethink how to do dose response level selection, as Dr. vom Saal said this morning. Currently the process is to conduct pilot studies to determine maximum tolerated doses. That dose is set at a terminally toxic level, then you step down a log order for the next level. From what was said during this morning's presentations, it seems we need a whole new definition of pilot studies down to the molecular level using in vitro data. EPA and the EDSTAC are going to have to deal with this.

The Office of Science and Technology has been inventorying research at federal agencies to find out what gaps exist so we can develop a rational research plan. EPA is putting about 10 million dollars into intramural and extramural research, which will looking at a variety of things related to biological effects. A lot of money is directed towards research on dioxins and PCBs. We need to do more work on test protocol design. We are trying to get a better handle on endocrine profiles in wildlife; we know a lot about mammals but very little about other species. In the environmental area (as opposed to the human health area) we are less concerned about individuals, and more concerned about population and population effects. We need to work on our ability to extrapolate what we are finding in individuals to the population level. The whole question of linking hazard and exposure is difficult, yet, we want to validate some of the information to improve our exposure and risk assessment tools. The United Nations Environmental Program, with the U.S. taking a lead has expanded the U.S.-based inventory. Other countries including Germany, Canada and the U.K. are now including data on their recent programs in that database. There has also been discussion on having an international assessment under the World Health Organization; that would probably be a two-year effort beginning some time this spring.

There are several major laws effecting chemical substances. When more information on these compounds is available, these laws will be used to establish regulations. First is the Food, Drug and Cosmetic Act (FDCA), which allows EPA to regulate tolerances of pesticide residues on food. The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) regulates the use of pesticides. The Toxin Control Act (TSCA) addresses commercial chemicals. The Safe Drinking Water Act (SDWA) requires EPA to set safe levels for toxic substances in drinking water. It is worthwhile to point out one area in the FDCA which will change, as much as any piece of legislation has, how we do business. It requires EPA to consider all routes of exposure when setting tolerances. Before, we established a tolerance on a particular food item and that was it. Now, we are going to have to look at the various pathways by which people are exposed, exclusive of occupation. We can look at drinking water or we can look at foods that might be contaminated by pesticides; so we're looking at the entire diet and more pathways. You likely will see more conservative estimates that we have had in the past. Now there is going to have to be an explicit tolerance level in children. If we cannot do that, an additional safety factor for setting tolerances of up to 10 orders of magnitude will be used.

The whole area of endocrine disruptors is controversial, and what we are seeing is science and public policy unfolding at the same time. Both are quite volatile. As information becomes available, we will see products voluntarily pulled off the market. One of the things that industry is concerned about is what they call "deselection of products". The public is going to feel that they cannot tolerate having products that are under this cloud of endocrine disruption. This is the way the EPA has wanted things to work anyhow. This is pollution prevention and it is putting things back in the marketplace where a lot of things belong. It is important that good information is passed on to people so that these kinds of decisions can be made on a rational basis rather than on the basis of misunderstanding or on the basis of fear. Regulatory policy will begin with the implementation of the EDSTAC recommendations. We have a long way to go. The priority setting phase is much farther along than the screening and testing committee. They are dealing with a lot more difficulty than the screening and testing committee. Once all of this is done, starting the validation program will begin. Head to head comparison to

some endocrine assays will be made. Ultimately regulations under TSCA, SDWA and FIFRA will be issued; we will be using the existing statutory framework for actual implementation.