

**Kristine Witt Interview**

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Interviewer: Sara Shostek

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*Beginning of transcript*

Sara Shostek: April 13<sup>th</sup>, and I'm interviewing Kristine Witt of the National Institute of Environmental Health Sciences. So you know that I'm tape recording our conversation, correct?

Kristine Witt: Yes, I do know.

SS: Great, thank you. So just to begin a bit about your experience here at NIEHS, what is your background? Your education and training?

KW: My education is a B.S. in Zoology, an M.S. in Genetics and then some work toward my Ph.D. but I never completed that would have been human genetics. So primarily a strong genetics background and a lot of that involved also genetic toxicology, or before – actually -- the term was formally coined.

SS: What was it called then?

KW: It was just genetics, but the toxicology of it was woven in to coursework and projects and things like that.

SS: When did you come to the NIEHS?

KW: I actually first arrived here the end of 1985 and I came on as a – on an interagency agreement with Oak Ridge Associated Universities -- let me know if somebody peeks their head in, if they want the room for anything we'll have to relocate. And I probably – gee I don't recall, but I was probably almost ten years in that position in the interagency agreement, through Oak Ridge. They are a DOE contractor.

SS: So you had been at Oak Ridge prior?

KW: No, I hadn't.

SS: No.

KW: Because there was currently a position open that was an interagency agreement funded position with Mike Shelby at NIEHS, and at that time Mike Shelby was integral to the genetic toxicology testing program here that was being developed. This was kind of in the early stages of development of the massive NIEHS NTP – National Toxicology Program -- genetic tox database.

SS: And what was the nature of the interagency program?

KW: The program was – there was Oak Ridge Associated Universities and Oak Ridge National Labs – these are two distinctly different organizations but located within very close proximity to each other -- both of those units had genetics groups – genetic toxicology groups. At Oak Ridge, it was more reproductive genetic toxicology. They did multi-generational studies looking at transmissible genetic damage or damage that somehow impacted reproduction. At Oak Ridge Associated Universities, they were looking more at the traditional in vivo mouse genetic toxicology endpoints, which were chromosomal damage endpoints. They were sister-chromatid exchange and chromosomal aberrations, and they got into micronucleus testing. It became a substitute for doing chromosomal aberration testing, which was arduous, time consuming, difficult on the scorer. The micronucleus test became a substitute for that and was a lot quicker and consequently less expensive to do. So they did those three endpoints and that's how I got involved because I had a genetic background. I did primarily clinical genetics work before I got here, but the last two years or so prior to my coming here, I became involved in genetic toxicology work out at the University of Utah. We were doing chromosomal aberration studies and sister-chromatid studies and micronucleus studies, so coming with a recent background made it easy for me to move into that open position here within the program.

SS: And then how did your career develop at NIEHS?

KW: As I said, I spent approximately ten years -- I have to actually go back and calculate, but it was around ten years I spent in that interagency position. And when I came to the NIEHS I obviously needed to quickly broaden my background because the NTP was involved in more than chromosomal aberration type assays; they were looking at other endpoints, they were looking at gene mutation endpoints in mammalian cell cultures, as well as in bacterial cell cultures. There was a little bit of work in unscheduled DNA synthesis for a while. That assay was done for just a very brief time. So during the involvement of my career I really had to become educated in all different aspects of genetic toxicology, not just as they pertain to the testing program developed -- or under development by the NTP -- but internationally. In the broad efforts in genetic toxicology, one of my primary responsibilities when I came to NIEHS was to prepare the genetic toxicology overviews that go into the NTP technical

reports. NTP has two-year bioassays. Are you familiar with those?

SS: Published what are called blue books?

KW: Yeah.

SS: I'm familiar with them, but I'm always happy to be reminded –

KW: Actually I can show you some of them. We have an entire library of every single report that's ever been issued by NCI, which began – where the program began and then NTP here at the NIEHS now has responsibility for that effort, and that is the cancer bioassay. The two-year studies in mice and rats, both sexes, of chemicals of interest, nominated either by government folks or an outside source for testing. Those reports contain an overview of what is known of the genetic toxicology of that particular chemical, either through testing done at other laboratories around the world or through NTP tests. It is still my responsibility to provide a literature review of the genetic toxicology data on the chemical as well as to prepare the data tables and summaries of the tests done under the auspices of the NTP on that chemical. So I spent probably six months, originally, learning everything I could about genetic toxicology; and then applying it. Both as a – primarily, in the beginning, with the reports and then, as I became more and more involved in the program, in using the knowledge I had acquired to help in discussions among the folks that were involved in the various genetic toxicology testing efforts for the NTP in determining what tests were most useful to the program. The mission of the NTP being primarily cancer, which of those tests best helped the program in its efforts to determine whether or not a chemical presented a risk of carcinogenicity, and which tests helped in understanding how that chemical might be promoting its effects. I actually, after the interagency agreement, was terminated about ten years after I first came here. I then became a contractor to the NIEHS and was employed by a contract company here in Research Triangle Park, that houses a number of contracts within the NIEHS, both pathology type contracts for the NTP related to the cancer bioassay work as well as genetic toxicology contracts. Just last July I became an NIEHS employee and so now I am even more integral to the genetic toxicology testing efforts of NIEHS and the NTP. I must say, in addition to the standard cancer bioassay work that's done in the genetic toxicology work, relating to that effort there's also and has been for years, a reproductive toxicology testing effort. Bob Chapin was in charge of that for years and during most of the time that I was here. Bob left maybe

three years ago or so, and Dr. Jack Bishop, who is on the east campus, took over responsibility for that. He is a research geneticist, and his expertise for years -- probably for most of the time he's been at NIEHS, he came about the same time I did, 1985, has been in reproductive toxicology, so he was involved with a lot of the contract work that was done through Oak Ridge National Labs; if you recall I said there were two institutes in Oak Ridge, one was the Oak Ridge Associated Universities and they did more of the traditional short-term tests looking at chromosome damage as it related primarily to cancer, and Oak Ridge National Labs did a lot of the heritable sorts of work, and Jack was in that for a long time and has been involved in some of the efforts, recently molecular sorts of approaches to looking at the types of genetic damage that may be induced in germ cells.

SS: Let me ask you a question about what you just said. I imagine that approaches to genetic toxicology testing have changed.

KW: They've evolved.

SS: Could you describe that evolution to me?

KW: One of the papers I suggested you become familiar with was the Tennant paper.

SS: Right, the 1987 paper in *Science*.

KW: The 1987 paper in *Science*. And there were actually a couple subsequent papers that built on that effort that were evaluations of the massive NTP database that was accumulated over, say, a ten-year period. When Tennant published that paper -- I think the beginning of that database was probably '82, '83, something like that, so it was maybe a five-year effort. Some of the tests may have gone back a little bit before that, but NIEHS was not one of the older institutes. I think we were -- I was here at the time, but I think it was -- what, '76, '77, something like that when the NIEHS was established.

SS: 1969.

KW: What, that's 1969?

SS: 1969, but it didn't have a building until much later. [Established as the Division of Environmental Health Sciences within NIH in 1966]

KW: Okay. So, just thinking back to the very early chemicals in the database, the earliest I recall having seen a date attached to anything was around '82-'83, and obviously chemicals must have been tested prior to that but that was probably before we had electronic databases. There are probably still hard copy files in somebody's archives somewhere with those early testing efforts, because actually our first salmonella testing paper -- compilation of maybe 200 or 300 chemicals -- was published in 1983, so obviously those chemicals must have been tested in the years prior to that. So those are probably still hard copy files somewhere, that have never been in the electronic database. But early on there was a fervent desire, and it's expressed in both the Tennant paper, and I think one by Shelby, as first author. It was perhaps in '86 or it may have been later in the '80s, that the goal had been and the thought had been surely there had to be a short test -- which is what we referred anything less than this two-year cancer bioassay -- a short-term test using fewer animals, costing less money and requiring far less time than everything that's involved in the two-year cancer bioassays; not just those two years, it's the preliminary studies, dose-setting studies and whatnot, leading up to that two-year, and then the several years after that for data analysis and evaluation. So we're talking about a six/seven-year effort, typically, to get out one of those cancer bioassays. So there must be a shorter-term test that could give us some very, very clear clues as to whether or not a chemical will be found to be positive in a cancer bioassay.

So the mouse lymphoma testing, that's an assay, the salmonella bacterial mutagenicity assay, the short-term chromosomal aberration tests and sister chromatid exchange tests in cultured Chinese hamster ovary cells. Those were four tests that were done on a number of chemicals in the NTP program, and for several years every single chemical that was of interest to the NTP and that looked like a reasonable candidate for testing in any or all of those assays. Another one was the drosophila sex-linked recessive lethal assay and reciprocal translocation assay. Okay, so those were both germ cell assays in fruit flies. Many, many, many chemicals -- in some cases hundreds of chemicals, particularly in the salmonella assay -- were entered into those assays and tested and data was collected, and a massive database was developed. And then the first attempts to correlate the data from those assays and from the cancer bioassays was made, and that first publication was the Tennant et al -- '87. And what that correlative study revealed, essentially, in a nutshell, was that the salmonella assay had the best predictivity. Tennant looked at a number of different things. Looked at -- I should have this paper in front of me -- predictivity,

sensitivity and two other ways of assessing the relationship between the short-term test data and the cancer bioassay. And it was determined that although useful information could be obtained from any of these tests regarding how a chemical might produce an effect, in terms of the rodent cancer bioassay, the best predictivity was provided by a positive response in a bacterial mutagenicity assay, which was the salmonella test.

SS: Which is also called the Ames test?

KW: Correct, because Bruce Ames was the person who first developed this test, and so it's often referred to as the Ames test. That was surprising to a lot of people, and it was counterintuitive to many people. Why would a bacterial mutagenicity test – bacteria are very, very simple organisms, genetically. One-celled organisms, they have a single chromosome that differs in many ways, structurally, from a mammalian chromosome. Why would that test be most predictive of a chemical's ability to produce cancer in a laboratory rodent, in mice or rat. However, the data were there. The data clearly showed the best correlation between a positive response – you don't get a lot of help from a negative response in a salmonella test. But to this day, 2004, the industry, the pharmaceutical industry, all international testing efforts to describe the activities of a particular chemical include the salmonella test as a required part of the evaluation of a chemical's activity and potential risk to humans from exposure to that chemical. So it is still today a positive response in the salmonella assay is a clear flag. It means this chemical is capable of inducing mutations in DNA, changes in DNA. Those changes may or may not result in an increased risk of cancer in laboratory rodents, which obviously - - the endpoint is not a laboratory rodent; we don't really care whether rats and mice get cancer from exposure to chemicals. But they're used, obviously, as tools to determine whether or not in certain circumstances, under certain protocols of exposure, a chemical can induce, in a mammal, lifetime exposure tumors. And then obviously we're extrapolating that test to humans. The other phase did not show good correlation between either positive or negative results in trying to predict whether that chemical would cause a tumor in rodents. Many of those assays were just overly sensitive, and in the case of the drosophila germ cell assays they were very, very insensitive. Very, very few chemicals caused positive responses in those drosophila assays. The ones that did we would refer to pretty much as bad actors. Those were typically fairly potent chemicals. If they could actually induce an effect in those two drosophila assays, they were probably going to cause an effect in something else and probably be fairly active in a rodent

bioassay, too. Consequently, they might carry an increased risk to humans exposed to it. But there were so few chemicals that were positive that it wasn't worth the effort of doing the test when you could get -- bacterial tests were so quick, relatively cheap and with the good correlation between a positive response in salmonella and a positive response -- in at least one sex, one species of rodents it became the gold standard. Now because bacteria are not mammals, people were both uneasy, and again you come back to this -- this is just not intuitive. Most regulatory agencies -- I can't think of one that does not also highly recommend an in vivo mammalian chromosomal damage assay of some sort. The most frequently used -- because it's least expensive, requires fewer animals and can be done the fastest, and requires the least extensive training of individuals involved in evaluating the cells -- is the rodent micronucleus assay. So currently the NTP maintains the capability of doing salmonella testing and rodent micronucleus testing on any chemical of interest to the NTP or members of a class of chemicals that the NTP determines -- even if these chemicals are not going to go into the bioassay they'd like to get an overall view of what's going on amongst a class of chemicals to be able to determine whether certain side groups or the position of a particular side group, how that might effect the genetic activity of a chemical. So we look at not only those chemicals that are of direct interest, because we know they're going to wind up going through a bioassay, but classes of chemicals. So the NTP now does salmonella testing, we do micronucleus testing. We have no longer the capability -- or we have not retained contractual ability to test in the mouse lymphoma assay, which is an in vitro mammalian cell mutagenicity assay. Nor do we any longer do drosophila testing. NIEHS has a very active drosophila genetic laboratory, but the questions that are asked by that laboratory are not genetic toxicology questions. They're exploring other --

SS: Who's lab is that?

KW: That is Dr. Jim Mason, and he might be a fascinating person for you to talk with because he was involved, in the very beginning, in the development of the drosophila testing capabilities of the NTP. He has now moved into -- I don't know exactly what he's doing, but I'm sure he's asking fascinating questions. He has quite a vital drosophila testing laboratory effort, and he also has been here for many, many years and could probably provide you a wonderful background of drosophila.

SS: That would be interesting.

- KW: That would be very, very interesting.
- SS: We were talking about the evolution of testing strategies.
- KW: Okay. So, salmonella has not changed very much over the years from the way it was probably 1989, 1992, around that time. Those were – for every year, two years, three years, Errol Zeiger, who was the individual who developed the NTP salmonella testing program, developed the protocols under which the NTP tests chemicals in the salmonella assay. The NTP now uses – used for many, many years, multiple strains. The strains primarily used are TA100, TA1535, TA1537, TA97, TA98, TA102, and TA104. Genetically, these strains are different, but some of them are related. Some are – TA98 and TA100 are basic strains. They carry two different types of mutations, and the histidine operon, which is what the salmonella strains are mutated – it's various areas within the histidine operon, and what we're looking for in the salmonella assay is taking a mutant strain which is not. I was just showing the audience what I told you before, what the major efforts were in NTP genetic tox testing when the program got underway in full swing and when lots of database development was going on. So chemicals were being run through all these assays and attempts were made to run the same chemical through all the assays, so you could compare the chemical and its performance amongst the different assays as well as look at the overall results in an assay database and compare how well the assay correlated with the sorts of things that were seen in the rodent bioassay.
- SS: Sister chromatid exchange –
- KW: Correct. Chromosomal aberration, Chinese hamster ovary cell.
- SS: Great. Thank you.
- KW: Unscheduled DNA synthesis.
- SS: Thank you.
- KW: These were the – let's see, what's the difference, past and present, past and present –
- SS: They're the same? No, they're different tests. In vitro, in vivo.
- KW: Thank you. Maybe I do need my notes going along with this. So here are the two drosophila tests I mentioned to you before, sex-

linked recessive lethal, that's looking at a gene mutation. Reciprocal translocation is looking at a chromosomal change. Mouse bone marrow chromosome aberration, sister chromatid exchange is the same type of endpoint, but looking in vivo in the entire animal, as opposed to in vitro, and obviously there are differences in vitro vs. in vivo. Number one is distribution of a chemical through -- the organism has to get to the target tissue before you can see a result. Number two, there's all sorts of metabolism that might be going on. So one would expect to see differences in vivo compared to the in vitro situation. The mouse and rat bone marrow micronucleus, that was used and is used now as pretty much a substitute for chromosomal aberrations, and I'll explain that test in a little bit. Mouse peripheral blood micronucleus, same endpoint, just -- red blood cells are made in the bone marrow, so we can look in the bone marrow or in the peripheral blood. There are a lot of advantages to using peripheral blood. It's a whole lot easier to obtain, and you don't have to sacrifice the animal to obtain peripheral blood samples. You can serially sample the same animal through time, or you can do other things with that same animal and just need to take a blood sample at some point when you're interested in looking at whether or not micronucleus frequencies in the red blood cells have been altered by chemical exposure. UDS is the unscheduled DNA synthesis in rat hepatocytes, where here we had an in vitro assay where we take cultured rat hepatocytes, expose them to a chemical and look to see whether or not we're having DNA synthesis occurring at times when we wouldn't expect it to occur in the cell cycle. That's indicative of the fact that damage was induced and now the cell has to repair that damage. So it's done either in vitro or it can be done in vivo, where the whole animal is treated, and then at some time point thereafter, hepatocytes are removed from the animal and DNA synthesis is observed at times when it shouldn't be occurring. So the currently active, we've gone through this, the salmonella, the two different micronucleus assays -- the peripheral blood and the bone marrow assay. The types of genetic damage that are detected by the assays that are currently used -- are detected by the assays and essentially we only have two assays: micronucleus assay and the salmonella assay. The Ames test, I used Ames test instead of salmonella because it's shorter, I can actually fit it in my column here. We look at single-point mutations -- and stop me if I'm saying things that you don't understand, because --

SS: So far I'm all right.

KW: Okay. Or we can look at very small deletions, not just a single base change but maybe ten bases taken out, or two or three bases taken – a very short deletion. These are currently used assays, not just within the NTP program. HPRT mutation – that is looking at a particular gene mutation in cultured mammalian cells, as opposed to bacterial cells. Again, that's looking at a single base change and it's also looking at small deletions. The mouse lymphoma assay, you've heard me refer to that, that's a type of lymphoma cell derived from mice that is cultured in vitro. That can assess base changes, small deletions, as well as large deletions -- chromosomal translocations and mitotic recombination. We won't go into the details of that, it's not really necessary. Chromosomal aberrations, say in Chinese hamster ovary cells in vitro, are going to look at large deletions or translocations. We're not looking at any single base changes there. Those are chromosomes and we're looking for fairly close changes in those chromosomes, structural changes. Micronuclei in bone marrow – they detect small deletions, large deletions and translocation. So in other words, structural chromosomal changes, and they also will alert us to changes in chromosome number. It's a phenomenon we call – or a situation we call aneuploidy. In other words, typically you have 46 chromosomes in a human. If you have 45 or 47, that's an aneuploidy situation. You've gained or lost a chromosome. The micronucleus test detects chromosome numerical changes. Then things that are involved – this has nothing to do with testing that we're doing now, but events that are involved presumably in the genesis of cancer. Those can be DNA base changes, they can be small deletions, large deletions, possibly – translocation, sorry. Possibly mitotic recombination, and suppressor gene activation can be brought about by any of these -- base changes, small deletions, large deletions, translocations, whatever simply by changing the gene itself or the location of the gene, the proximity to other genes within the genome itself. So looking at all of these things, pretty much the Ames test and then the micronucleus test cover almost every one of these possibilities. So even though we have currently restricted our program to micronucleus testing and Ames testing, we are not ignoring lots of different possible mechanisms that might be involved in carcinogenesis.

SS: So may I ask you a question?

KW: Certainly.

SS: Since you used the word mechanism – I hear a lot of talk about mechanism-based toxicology and bringing mechanisms into the NTP. How does that intention relate to these testing programs?

**KW:** That relates to these testing programs in two ways. One, indirectly, because we are looking at genetic changes by doing our two tests that we pretty much restrict ourselves to. By looking at the results of those two tests, within the context of other information known about the chemical, whether that might be results of the bioassay or additional tests that the NTP is doing or knowledge that we acquire through other laboratories around the world, we can infer mechanism. The other – in other words, these results might be consistent with a proposed mechanism, okay? The other way is that the NTP genetic tox testing program has been authorized, through approval of a concept review, to expand beyond doing these two assays and looking at certain mechanism-based endpoints. That effort is currently under development. We're looking at ways to proceed by right now trying to learn as much as we can about some of the different assays, say assays that measure induction of apoptosis. Apoptosis is programmed cell death, and it's something that is involved in cancer because it's believed that mechanisms that trigger apoptosis are overridden by cancer cells, and they lose the ability to commit suicide, and therefore damaged cells continue to divide.

So we're looking at how we might develop useful assays. We're looking at apoptosis, for example. We're looking at whether or not there are different protocol designs for the current assays that we do that may give us more mechanism-based information. This is an ongoing effort, and it's not a cop out, we're just trying to be very careful. Having learned in the past that jumping on a bandwagon is not necessarily the way we want to go because things – new tests, new protocols, whatever, can appear to be extremely promising and exciting at the outset but as soon as a little bit more work has gone into an assay or thought or looking at how that might fit into a broader picture, turns out that we might be getting interesting information, but what to do with that information is not really clear. So we would like to have a real careful approach to how we proceed with that, and I know that may not be very satisfying at this point but we spent almost a year already looking carefully at how we might use mechanism-based science, as opposed to just testing, and we're exploring on paper a lot of different approaches, but we've not yet put forth a proposal for testing.

**SS:** And when you have mechanism-based data from an assay, what does that enable you to do that having more the phenomenological data does not enable?

KW: Okay, well once we know how a chemical induces an effect that we're measuring, we can determine a couple things. We can determine this is the only mechanism by which this chemical operates, or can it operate through different mechanisms under different situations. And then number two, when we have clearer ideas of how a chemical interacts with biological molecules, we can determine, hopefully, whether or not those mechanisms are active in an in vivo situation. In other words, if we see something occurring in vitro, is that relevant to what's occurring in vivo? In vivo, you're going to have more complex metabolism. Are you going to generate the same kind of active intermediates or endpoint metabolites and will they be able to induce those same effects? So we hope to get more relevant information and help in -- ultimately in risk determination. I mean, that's the ultimate thing. Can we modify exposures in a certain way so that we're not going to generate those harmful intermediates or endpoint metabolites? I guess we'll have clearer ideas of what to do with that information when we get broader and more in-depth databases that are mechanism-based databases, but the hope is that the more you know about a chemical and how it operates in vivo, the better you're going to be able to determine what the ultimate risk is, or how to mitigate that risk. What you can do to block that chemical action.

SS: That's very helpful, thank you.

KW: Okay the salmonella assay, I told you before it's used everywhere. Industry, all international testing organizations, whatever. It has got the highest predictivity for rodent carcinogenicity -- a positive response in this assay with the highest predictivity. A variety of different tester strains are used. They are all genetically different, okay? Slightly different. They all have mutations within the histidine operon, but slightly different mutations. So this way, using a number of genetically different strains allows us to provide more information, more opportunity for a particular chemical to induce a mutation, basically, because some chemicals may be better suited to looking at larger targets -- we call them frame-shift mutations. They are going to target the small deletions, small shifting of certain strands of DNA. Others are going to alkylate a particular base and cause a base change. So different mechanisms.

Currently the NTP is testing in TA100 and TA98, which are the basic strains from which these other strains are derived. We have the capability of looking at a number of different strains if we have a reason to do so. If we get a positive response in TA98 or TA100 under different testing situations, that's enough. Basically, our

mission is to determine can we induce mutations in salmonella. If the answer is yes, we'll move on. And if another agency comes back and says, "We want more information. We want to know a little bit more about how broad the capabilities might be of this chemical. Are there other strains that are sensitive?" And then that may give us a little bit more information about how a chemical may operate, although Errol Zeiger, who's the person who developed our salmonella testing, in one of his recent papers -- I can get you the quote if you need it -- stated emphatically in his review of the thousands of chemicals that have now gone through the salmonella assay, the most important endpoint is does it induce a mutation, yes or no in a strain, under a situation. We'll get to activation, whether or not a chemical required metabolic activation. There does not appear to be any relationship between strength of response or broadness of the response across a number of strains, and carcinogenic potency, if we can use that word of a chemical. So if you get a yes or a no answer, there doesn't seem to be a whole lot gained by getting additional answers. There may be certain instances where gaining additional answers, because we're doing a class of chemicals and we want to see how responses are going to differ with certain side groups added on to a parent molecule, but in terms of getting any more information, predicting the carcinogenicity of a chemical in the rodent bioassay, there's no more to be gained.

SS: Okay.

KW: So at this point we're testing TA100, TA98 with and without what we refer to metabolic activation. Now that is because when you take a chemical and put it in a person, the liver metabolizes that chemical. Bacteria have no liver, therefore what we do is we get liver enzyme extracts from rodents that have been -- typically have been what we call induced. In other words, they are pretreated with a chemical that heightens the liver enzyme profile in that rodent, so that we're going to really have that liver revved up, lots of metabolizing enzymes are going to be produced, so that when we go in and extract those enzymes we've got quite a pile. And then we take that -- it's well defined protocol, in fact which by commercial S9 is what it's called, and then add it to your salmonella strains in various amounts and then you test your chemicals. You test it with and without activation. That lets you know whether or not to anticipate that a chemical metabolite or an intermediate is actually inducing the effect. Because if you get a response without liver S9 then it's presumed, since the bacteria really doesn't metabolize the way mammals do, that the chemical is a direct acting mutagen. If you need to add S9 liver enzymes to

the salmonella strains before you detect mutagenicity, then metabolism of that chemical to an active intermediate or an end metabolite is necessary to produce the mutagenic response.

This is the slide that just describes S9 -- what it is, where you get it from. There are P450 enzymes in the liver. Most people have heard that term anymore. This is just a schematic actually showing how you do this very simple assay. Essentially, you mix your bacteria, your media, your test chemical with or without the S9 depending upon if you're using activation or not. Mix it all together and remember I told you we're looking at mutations within the histidine operon, so the bacteria normally can manufacture their own histidine. These are mutant bacteria that we're testing in the salmonella assay. They have got a mutation somewhere in that histidine operon that prevents them from manufacturing their own histidine. So they have to be given histidine in their growth medium. So we take these bacteria, we expose them to a chemical, we grow them out on a histidine deficient plate, so that if we have no mutations occurring, we're going to get only a certain number of mutant colonies growing on that plate. There's a little bit of histidine in there -- trace amounts of histidine in this agar in which these bacteria are grown -- to allow them to undergo a couple of cell divisions, because you have to, what's called, fix a mutation. The DNA has to divide, replicate in order for that mutation to be fixed. Then you can get mutant colonies to grow. So you give a little bit of histidine to allow a little bit of growth to occur before it runs out of histidine and nothing's going to happen then unless you've got a mutant cell growing up into a colony. So you've got a certain amount of background. If your chemical is a mutagen, and this is hard to see on this printout.

SS: No, I can see the difference.

KW: There's an increase in the number of mutant colonies. More chemical, higher dose, look at that. This is such an easy, straightforward test. Typically we look for a dose response, we test at least five doses of chemical maybe more, and you should see a clear dose response with increasing number of mutant colonies if we've induced a mutation.

SS: That's very clear.

KW: Very easy, very straightforward. We do automated plate counting, whatever. So this is just -- it's such a quick and easy assay to perform. It's just unbelievable that such a quick and easy assay

correlates that well with – but we’re looking at a genetic event, and cancer’s a genetic disease. Not all mutagens are carcinogens, not all carcinogens are mutagens -- there are other mechanisms by which you get cancer. But there’s a pretty good correlation between mutagens and carcinogens.

SS: And the other mechanisms are promotion, right? Non-genotoxic effects?

KW: Right, right. You’ve got an initial hit -- something occurred, and then you’ve got a chemical which is going to stimulate cell growth, for example. So if you’ve got a mutant colony, and it’s – or a mutant cell, whatever, and all of the sudden you do something to that tissue or that group of cells to really promote their turnover, more than likely you’re going to promote the growth of cancer, because the faster a cell turns over continually, the greater the chances of an error occurring, or the less time there is to get that error repaired. And what you have is you’ve got signaling going on within the cell, saying, “Wait a minute, we’ve got a mistake here. Stop, everybody hold. We need to repair this DNA.” Repair, okay? But then you’ve got this other stimulus saying, “Uh uh. Divide, divide, divide, divide.” And these get out of whack, and so the request to divide overrides the request to halt all operations and fix what’s gone wrong, and that is a mechanism by which you can generate cancer.

SS: Let me ask you more about that because one of the things that I’ve been studying down here is the development of transgenic mouse models as bioassays. Will that appear in this at any point?

KW: That is actually one of the organisms that we do the micronucleus test on. That’s pretty much our involvement, it’s just that they are doing a number of these tests, and we are routinely doing peripheral blood micronuclei on these animals, frequently at various stages during the exposure. They may expose those animals 26 to 39 weeks, something like that. So we may actually have micronucleus data at 4 weeks, at 13 weeks, at 24 weeks, at 39 weeks, which is very interesting because we can see what is happening to that micronucleus [inaudible] through time, and are there any differences. And also I think the validation effort with those transgenic strains is using, either simultaneously or used previously, that chemical in the B6C3F1 mice that are standardly used in the bioassay, so you get comparative data between the transgenic and the traditional B6C3F1 mouse. And so we have got micronucleus data for some of those chemicals in both situations, and so we have comparative data there. There hasn’t been any

formal analysis yet of that data. I'm sure there will be when the testing effort is thoroughly analyzed and compared to the bioassay. That is our involvement. We're not in any way, shape, or form, directing that testing, or inputting in to study design or anything like that, but we are getting blood samples from those animals and routinely doing micronucleus studies on them.

SS: My understanding is that at least one of those animals, the TGAC mouse, was developed in part because there was a perception that the NTP needed a promoter assay.

KW: You would really have to talk to the folks that are involved more intimately in those transgenic assays.

SS: I guess to ask the question differently, has it been a problem or has it been a challenge for the NTP that a good promoter assay hasn't been available?

KW: Promotion is obviously a mechanism through which cancer is generated. I can't really answer that question because I'm just not involved in the bioassay aspect of that and it would be better to get a historical perspective from somebody who has been involved in that for a long period of time. Probably also because of the fact that up until a year ago, I was either a contractor or, on the interagency agreement, there were some restrictions on my involvement on the NTP because I needed to stay within the guidelines or the directives of my employment agreement, and so I focused more on just the genetic toxicology, as opposed to the broader NTP efforts. Now that I'm an NTP or NIEHS employee, I'm really involved in a number of things that I can see gain quite an appreciation for all the different efforts that are going on, but since I don't have that historical background, and that – I could probably be – mislead you or just because of the fact that I've got a lot of holes in my background there, so you need to talk to the folks that are experts in that. Have you talked to Jef French, or do you have –

SS: I have, thank you.

KW: Okay, okay because Jef French's name comes to mind when you say TGAC.

SS: Right.

KW: There are modifications in terms of protocol so that we can test gaseous – the salmonella assays – so that we can test gaseous

chemicals...volatile chemicals, things like that. The pre-incubation modification step is very simple. All it does is allow us to hold the mix for 20 minutes or so and allow greater exposure potential of the chemical in the cells before we pour it onto the plate. So these are just very basic protocol modifications. This is the – just a picture for the audience to show them how we stack plates in a dessicator and then we feed in either air through a tube that's got the chemical that's a vapor in it, or we put a volatile chemical below and allow it to diffuse through a chemical below and allow it to diffuse through a dessicator but we trap the chemical in so that we get good exposure of the plates.

We evaluate the data, and we determine whether or not a chemical is a mutagen. If we see a reproducible dose-related increase -- I showed you those plates, and clearly you can see dose-related increases -- we've got a mutagen. We may characterize some chemicals as weak mutagens if we have a dose-related increase, but the magnitude of that increase is less than twice the background number of colonies. We may say, "Yeah, this is clearly mutagenic," but there is a difference. So basically we find out whether or not our chemical's a mutagen or a non-mutagen, and a positive response in any strain -- remember I said we can use multiple strains -- under any activation condition is sufficient for an overall positive call. So we don't require like two different strains and with and without S9. Then there's the rodent bone marrow micronucleus test, okay? That's the one I was telling you that -- rodent bone marrow or peripheral blood are our two micronucleus test capabilities. The rodent bone marrow micronucleus test detects induced chromosomal damage in erythrocytes, red blood cells. We can detect either a change in chromosome number, or structural damage. Do you know what a micronucleus is?

SS: No.

KW: No, okay, so –

SS: Actually I was just reading that.

KW: All right. So when a micronucleus – when a cell undergoes some sort of damage, either a spindle fiber's disrupted, its centromere in a chromosome is structurally altered or something so it doesn't move correctly during cell division, or if a piece is broken off or whatever, when the cell divides and forms two daughter cells, that whole chromosome or that little hunk of genetic material that has broken free is oftentimes lost. It's not properly incorporated

because it hasn't moved to one of the two poles when the cell divides, and what happens is when the cell goes back in to interface then it forms a nucleus membrane around its genetic material. That little piece or whole chromosome that's left forms the same little thing, so it's literally a micro-nucleus sitting inside the cell. It's easily detectable.

SS: Great.

KW: And the reason that the NTP uses erythrocytes is because – and see this on the slide is florescent staining and whatnot so you can see how easy it is to detect it, but this is exactly what happens. As you've got a piece or a whole chromosome -- in this case it's a piece that's broken off, and here's your two daughter cells, you'll form a cell membrane in between there, but there sits your little micronucleus. And actually, this picture is a little misleading because this micronucleus will look exactly like that – the staining characteristics, the texture of it etc., is exactly the same, it's just much smaller. So it's very easy to see. You go under a light microscope, they use fluorescent stains and there sits your little micronucleus. There sits one, and actually it looks like there's another one right there. So it's so quick. You can scan, literally, thousands of cells and in the case of an erythrocyte – let's see if it – no I don't have a picture. These are just additional pictures and we'll just – without having them on an overhead they're harder to see. The erythrocyte is kind of unique, in that it doesn't contain a nucleus. So it did at the start -- the stem cells do, but as the erythrocyte matures, the last thing it does before it becomes a full-blown erythrocyte is it pushes out its nucleus. Erythrocytes are terminal cells, they cannot divide any further. So if you're scanning a whole bunch of erythrocytes, say these are erythrocytes here, they will have nothing that fluoresces – we used a DNA-specific stain. But the thing about micronuclei in erythrocytes is that if, prior to that last cell division, a micronucleus has been formed due to a structural or numerical problem, the micronucleus apparently too small for the cell to sense its presence, so it is not pushed out of the cell with the main nucleus. It stays behind. So erythrocytes are extremely amenable to scoring micronuclei. You can score a thousand erythrocytes and two of them are going to have micronuclei. And they'll just stand out so easily, so that's why we score erythrocytes. In the bone marrow of mice and rats, we can only score erythrocytes in the peripheral blood of mice. The reason is when a damaged erythrocyte moves down into the bloodstream, the spleen, within just a couple hours -- as the blood circulates it goes through the spleen on one of its many trips, and the spleen can detect whether or not there's damage to a blood cell,

and it will remove that blood cell from circulation. So in rats they have very, very efficient spleens, as do humans, and they can instantly pick up on a damaged erythrocyte, they'll remove it, so by the time we go to look in the peripheral blood for micronuclei erythrocytes, they're not going to be there.

SS: That's interesting.

KW: But mice have very, very inefficient spleens, and so the damage that occurs in the bone marrow moves out into the blood and is not removed, so we can do peripheral blood on mice, which is really, really nice. These are just more slides that show how the difference between an anugin [spelled phonetically] and a clastugin [spelled phonetically].

Why are we interested in a chemical's ability to induce micronuclei? Okay, so it's a chromosomal change and most cancers are characterized, actually, by chromosomal changes that occur at some point along the way -- chromosomal changes are characteristic of almost all tumors. Aneuploidy -- chromosomal changes in germ cells, we're not talking about cancer at a point now, we're talking about germ cells -- are major causes of birth defects and spontaneous abortions, and other sorts of adverse reproductive outcomes. So actually, looking for chromosomal damage as a result of exposure to a chemical gives us information not only about whether that chemical might have some carcinogenic potential, but it also alerts us to the fact that if that chemical gets to the germ cells, that there may be some reproductive consequences to expose you to that chemical also.

It's very relevant to know whether or not a chemical can induce chromosomal damage in vivo. So this is just an overview of the standard protocol for doing a micronucleus test. This is the bone marrow test. Basically, you treat your animal -- we typically treat three different days, because if we're somehow interfering with the dynamics of erythrocyte production, by administering a chemical once, we would probably have to use more animals because we would have to harvest the bone marrow at different times, so if we just take our usual number of animals and treat them multiple times than just harvest once, we will have pretty much taken into account any kind of changes in erythropoiesis that may have been induced by the chemical, or requirements for a metabolism of the chemical that might take a while to generate the active intermediate or metabolite. So this is, again, just an overview -- we treat the animal then we remove the bone marrow, we look for micronuclei.

SS: Okay.

KW: Very easy. And again, this is – this looks really pretty in color. The peripheral blood micronucleus test, we treat the animals for a longer period of time. We routinely incorporate that test in our 13-week toxicity studies. Those are studies that precede the bioassay, they're usually dose-setting studies or whatever, so routinely at the end of those 13-week studies we get peripheral blood samples, we make slides and they're sent to the lab and scored for micronuclei. That way on the same animal where maybe some immuno tox stuff is done or some neuro tox stuff is done or whatever other sorts of toxicology endpoints are assayed in those animals, we can in the same animals also get micronucleus information, which allows us then maybe to better correlate everything, because it's exactly the same animal, so I have a separate test being done. Same thing – the animals are treated, get a blood sample, make a slide, stain it, look for micronuclei. Very, very simple. The evaluation – we look for both a trend – in other words, some sort of an increase, overdoses, because there's multiple doses always used -- and we also look at each dose level and we compare it back to the control. We use fairly stringent statistics to determine whether or not a chemical is positive, and if we get either – or both a positive trend and at least one of the doses is significantly different, we say, "Okay, this chemical is capable of inducing micronuclei." The acute tests are always repeated. These longer-term peripheral blood tests are not because they're piggybacked on to some other test and those tests are not repeated. So usually we'll have male and female. We feel a little bit better if we see the same response in males and females. But if clearly one sex responded differently than the other we called the chemical positive, say in females, negative in males.

In this particular talk, I looked at different sorts of results comparing salmonella, micronucleus and then the carcinogen assay. Are all of these discordant – these were all discordances. Because somebody had asked a question once, "If you're positive in salmonella are you automatically positive of micronucleus, and how does that relate to carcinogenicity?" An example here is three NTP chemicals. They were all very nicely positive in salmonella, they were all very nicely negative in the micronucleus test and they were flaming carcinogens. Okay, that's okay, these are strong gene mutagens; causing a gene mutation is definitely a way that you can get cancer, okay? So there's a discordancy among the three tests, but nothing scientifically odd about that group of chemicals and those results. We can have the converse.

Aniline-benzene is a very good – that's a human carcinogen.  
Phenylthaline was an ingredient that was in ex lax –

SS: Laxatives.

KW: Exactly. Aniline is a dye, or the basis of a lot of different dyes. These are all real good carcinogens. There's a dispute as to whether phenylthaline's a human carcinogen, but it was clearly an animal carcinogen. None of these are positive in the salmonella tests. They don't cause gene mutations. But they're all positive in the micronucleus assay. Benzene is just a flamer and phenylthaline's a very good inducer of micronuclei, particularly in the longer term peripheral blood studies.

SS: Let me ask you about phenylthaline. I interviewed June about phenolphthalein, and she used the transgenic mice, right?

KW: Right.

SS: She used P53 mice --

KW: Right.

SS: To show that phenolphthalein induces mutations in the P53 gene.

KW: It does, and it also has a very strong micronucleus response of those P53 mice, also.

SS: Why would something cause mutations in P53 that wouldn't show up in salmonella?

KW: The P53 – first of all, benzene as an example requires an extensive metabolism before you get to the chemical. I can't remember now – phenolphthalein – what the metabolic profile of that thing is –

SS: But that would be the answer, that it requires activation?

KW: That would be the answer, and some kind of – and then plus, also you realize that those animals have got one hit already, so they're damaged in terms of DNA repair. So if something is breaking DNA, they've already got one hit. If breaks occur in areas that might affect the P53 gene, you could also suppose that you might have something going on there. And P53 is that gene that has to do with DNA repair. So I think, basically, it is metabolism because I know the same thing is going on with the animal too.

SS: Great, thank you.

KW: In fact, I said phenolphthalein is a very good inducer of micronuclei in the longer-term peripheral blood in a 13-week study with the B6C3F1 mice, that thing came out flaming positive, and there was no other indication at that point that anything was going on. So they made the decision to go into the bioassay because there was a positive micronucleus response. One thing I didn't say about the peripheral blood micronucleus test – a preliminary analysis of those tests, which was published in – I can give you that actually -- showed that although very few chemicals are possible in the peripheral blood micronucleus study in the NTP thing, those that are are all carcinogens. So the fact phenolphthalein came up positive -- strongly positive -- in male and female mice in 13-week studies was a real eyebrow raiser, and so they went ahead and they did the bioassay, and lo and behold it was as carcinogen. And when June did some additional longer-term studies in the P53 mice, we also did micronuclei in those mice, it was just amazing micronucleus levels. But the acute bone marrow studies we did with phenolphthalein were a real problem trying to get – not trying to get micronuclei – there was something going on with the metabolism. We did a whole series of tests with phenolphthalein – administering it in food pellets, fasting the animals beforehand – it seemed that you needed to get long-term, low-dose exposure -- there's something going on metabolically with that. So that's why it was negative.

SS: I hate to do this, but in the next five or ten minutes, I need to go to the other campus and do an interview over there.

KW: Okay, that's fine. Well here are your notes. They've printed.

SS: Fantastic.

KW: You've got the slides on here, too, I think. Yeah, so you can match those all up. Again, these are just – and here you've got non-genotoxins inducing cancer, here you've got genotoxins -- look, ascorbic acid, vitamin C; no cancer. So you can get every possible combination, but there are reasons for this. We're not just going, "Wow, are we surprised." Hopefully, we have some understanding of what's going on with these chemicals.

There are new assays being considered by the program. In fact, we are currently doing validation assays now, looking at what we call kinetochore staining, that's using a micronucleus test where we're staining the micronucleus with a second fluorochrome to see

whether or not there's a centromere in that micronucleus. If there is, that's strongly correlated with an entire chromosome being in there. So it gives us an idea of how that micronucleus got there. Is it an aneuploidy event, is it chromosome damage?

We are doing this. Flow cytometric analysis of mouse or rat -- this is the big breakthrough, because we can label the erythrocytes with a marker for a protein that only appears on brand new erythrocytes right fresh out of the bone marrow. We can grab those within one to two hours before the spleen's gotten to them in the rat, and evaluate them for micronuclei. That gives us the possibility of also looking at rats in the 13-week studies. We've never been able to do that before.

So we're doing these currently right now -- big validation studies for both of these. We're also doing comet assays, which looks for DNA damage in a single cell -- all sorts of DNA damage, kind of indiscriminant DNA damage, as an adjunct to some of these other endpoints that we're looking at. But we can do this in the same animals that we're doing micronucleus testing and in a variety of tissues, which is nice. We can look at liver, we can look at kidney, we can look at stomach, we can look at brain. These are just more slides talking about the types of DNA damage detected in the common assay -- a very, very basic outline of what's done in the common assay. Again, we can use the same animals that we use for a micronucleus test. It's just at the end things are done differently with those samples than the blood.

We can also do common in the blood, too. This is how it gets its name. We're looking at something that actually looks like a comet. This is the cell, it's moving through -- it's being electrophoresed through a gel and the DNA that's damaged is going to be moving at different rates, and it looks like a comet. There's a computer program that visualizes this and measures this as well as volume and from that is computed the degree of DNA damage. Participants, ILS -- these have changed. We have a new contract now, and Bill and I are now project officers. Bioreliance is finishing up its salmonella testing for us. SRI is no longer a contract, not because they're not fantastic -- Christine Morolmans [spelled phonetically] is one of the pioneers in salmonella testing. SciTech is doing our kinetochore work for us, and they're currently doing our salmonella testing for us. ILS is our overall contractor, they're here in the park. And again, just additional things -- I went back to the beginning.

- SS: That's great. That's so helpful. Thank you so much. I wish you were a biology professor. You're an incredible teacher.
- KW: Well thank you. Thank you. Had I – I should have actually refreshed my memory on this last night. We have got different versions of this, I think.
- SS: Is there anything –
- KW: I'll just put all this together and you can sort through it because I know you have to run. I think we've got two versions of the actual program and here's the one with the notes, and that's probably the one you're going to use but we'll go like this.
- SS: Is there anything I should have asked you or that we should have talked about that we haven't touched on yet?
- KW: If you're talking about the history of the program, I don't think so. The one thing that I need to provide you if you don't already have them are the follow-up studies that were done to the '87 Tennant paper.
- SS: I don't have them.
- KW: Okay, because I'm believing that there were two – there may have only been one, but I think there were two follow-ups where they took additional chemicals that came off like, say, in another two years from the testing program and they did the same sort of analysis, comparing to the bioassay, and it confirmed and refined the predictivity, so you might want to have those just for completion.
- SS: Okay. I can ask Ray, also, if that would be easier.
- KW: Oh yeah, oh absolutely, sure.
- SS: I will be back here in July to do a presentation with Ray.
- KW: Tennant?
- SS: Yes.
- KW: Okay, okay --
- SS: The history piece, and then he'll talk about state of the art testing.

- KW: Yeah, moved on into the microarray work and all of that.
- SS: Right, and actually just, in a couple of minutes, is there a way in which these tests and these protocols have led to toxicogenomics and microarray? Are they related? And Mac and I were talking the other day about the difference between looking at gene damage and looking at gene expression.
- KW: I don't know that micro – Ray would best be able to explain how microarray arose. But as technology developed we gained very, very rapidly an ability to go from taking days or hours to sequence or to analyze gene activity or something to – all of the sudden the capability of looking at a thousand genes and seeing which ones got turned on and which ones got turned off, and with the frustration of not having been able to devise a short term test -- and I think they probably won't ever be able to, it's just my personal opinion. I think cancer's a very complex disease, and every cancer is a different disease, it really is. So I think it's too simplistic to say that surely there must be some quick test tube thing that can predict – so this was very frustrating, that they couldn't come up with some way of at least narrowing down the candidate chemicals, although salmonella does that very well by saying that if you have a positive salmonella, that probably this is a carcinogen and let's not even deal with this chemical further. Let's look at the ones that weren't positive in salmonella. I think this really spurred, as soon as somebody realized that there was a possibility of assaying a lot of genes for activity all at one time, all of the sudden this basic desire to find some way of quickly gaining a profile of a chemical's activity in an organism blossomed back up again, and the thought was, "Oh my goodness, here's a way of doing this." There are probably two camps at least, right now: one that thinks microarray is the greatest thing since sliced bread and is going to be the answer for everything, because look, here we've got thousands and thousands and thousands of genes turning on and turning off; there is the other camp that says, "Uh huh, you do and you're generating tons of data and nobody has a clue what to do with any of that data." And they are entering it into databases but nobody yet has figured out what to do with it. I have a lot of confidence that somebody will, or somebodies will figure out what to do with all this data and what it means, but right now they're just looking at patterns. What happens when this chemical and this tissue or this organism or under these circumstances enters into the picture? So again, it's back to trying to find a tool that's going to give us an answer without having to go through the black boxes of the bioassay. Organism, chemical in, tumors out. What happens in between and what does this have to do with people? It's a leap

from the bacteria to the mouse and the rat, and it's at least as big a leap to a real life human exposure scenario. People are usually not exposed to high-dose lifetime exposures, okay? It's either an acute once in a while or it's low dose continual or maybe for ten years, or during a certain period of development.

SS: Right, occupation, right.

KW: Right, seven hours a day. The exposure scenarios in humans.

*End of transcript*