Earl R. Stadtman, Ph.D.

This is the second interview in a series on the career of Dr. Earl R. Stadtman. It was conducted on January 30, 2001, in his office on the second floor of Building 3, National Institutes of Health, Bethesda, Maryland. The interviewer is Dr. Buhm Soon Park.

Stadtman: I think we already discussed the cascade system. Right?

Park: Yeah.

Stadtman: One of the rather important contributions that came out of these studies on glutamine synthetase was some studies of the immunochemical characteristics of adenylylated enzyme, and that stems from the fact that glutamine synthetase is a dodecamer made up of 12 identical subunits. And each of those subunits can be adenylylated on a tyrosine residue, which alters the feedback control and allosteric regulation of the enzyme. Bob Hohman[1] was a graduate student at Maryland under my supervision, and I suggested that he get antibodies against the adenyl group on glutamine synthetase and then study the properties of partially adenylylated enzyme. So those immunochemical studies were rather interesting because they showed very clearly that in order to get interaction, you had to have more than one adenylyl group per molecule. That it was especially of interest that if you had partially adenylylated enzyme, then the closeness of the adenylylated subunits within a given molecule would alter the interactions with antibodies that he was able to prepare against the adenyl group. So he made a, I thought, a rather significant contribution to the field of immunochemistry and polymeric

1 Robert Hohman

enzymes.

Park: Did you have graduate students in your lab often?

Stadtman: I did at one time. I had--he was a graduate student in my lab, and before him there was Bob Burton[2], who was way back. He was a graduate student. He got a master's degree with me, not a Ph.D., at Georgetown University. And then I had Emily Shacter, who was a graduate student from Johns Hopkins, and she was an exceptional student, did a lot of basic work on this cascade, phosphorylation/dephosphorylation cascade systems. And then there was a woman from Johns Hopkins that worked jointly with Rodney Levine and with me for her graduate studies. I'm trying to think of her name [Jo Ellen Roseman]. But anyway, those were three rather outstanding... Bob Homan, I just mentioned, got his degree at the University of Maryland. But I was--at one time or another, I gave a course at Georgetown University. I gave a series of lectures at Johns Hopkins University, in their graduate program. Each year I gave a series of lectures on cellular regulation. And at the University of Maryland, I was a visiting professor for several years in the microbiology, and I gave a course there for several years running.

Park: So you have been involved in teaching?

Stadtman: I have been involved in teaching. I taught a course on three different occasions in Camerino, Italy, where I was invited to give a course on cellular regulation. I was a selectee of the American Chemical Society to

[2] Robert M. Burton

make a--I forget what they called it officially, what they called the program, but it was a program where you went from one major university to another in this general area and give lectures.

Park: Do you like teaching and giving lectures?

Stadtman: I like teaching, yes, and, as I pointed out to you last time up there, a series of notebooks that contain some of the lectures that I gave.

Most of this is cascade stuff, which is summarized in these reviews that I've pointed out already. This is the Hohman paper I was telling you about, which shows the epitope density in immunochemistry.

Well, now then, I'm going to shift to a new area of discussion.

After spending many years in this field of cellular regulation by phosphorylation/dephosphorylation, adenylylation/deadenylylation, of–groups on proteins, either serine or threonine or tyrosine groups, it occurred to me that there was really very little known about what regulates protein turnover in the cell. It had been pretty well established that it's under regulation because, depending upon the diet, you would find certain enzymes were turned over, disappeared, if you will, levels decreased, and other levels of proteins would increase, and there was a lot of evidence showing, using labeled proteins, that there was constant degradation of proteins. But the question arose, why the--how was the specificity determined? What was it that governed the regulation of protein turnover?

And so we carried out some studies where we utilized *E. coli*, which is

known to undergo rather rapid protein turnover when it goes into, when it's starved of a given nutrient. So we studied the activities of 15 enzymes in *E. coli* when the *coli* were starved either for a nitrogen source or a carbon source. And what we found was that, out of those 15 enzymes, there were five of the enzymes that went up, five enzymes went down, and five of the 15 remained the same. But they differed. The ones which changed and the ones which remained constant varied depending upon whether it was nitrogen starvation or carbon starvation that the organism was exposed to. And this led us to look more carefully at what happened to those enzymes that did decrease during starvation, and among those enzymes was glutamine synthetase, which we had already done an enormous amount of work on, so we concentrated on glutamine synthetase. And what we found was that when extracts of *E. coli* were made from the phase where nitrogen starvation had occurred--we made extracts of those organisms. Then the, at least glutamine synthetase--we didn't look at all of them, but glutamine synthetase level dropped and we found an extract that you added, that this decrease in glutamine synthetase activity was dependent upon oxygen, it required a metal, and it also required an electron donor such as NADH or NADPH, and these are classical mixed-function oxidase systems that had been described several years before by a group here at NIH[3]. So we were rather excited by this, and we studied in some greater detail the mechanism of oxidation of

[3] Sidney Udenfriend and colleagues

glutamine synthetase by a mixed-function oxidase system that consisted of ascorbic acid as the electron donor, a metal ion, and NADPH. And what we... Well, I'll come to more of that later.

But first I might point out that we found a number of enzymes in this cell were susceptible to oxidation by this mixed-function oxidation system. And so I suggested to another postdoc[4] that came from, originally from Great Britain, that she might look to see whether or not there's a protease that could discriminate between oxidized and non-oxidized proteins, and she found, in fact, that there were three proteases in extracts of rat liver that had the ability to discriminate. One of them was the 20S proteasome or multi-subunit protease, and another was a calpain--that's a calcium-dependent enzyme--and the third one was in the class of--what do they call them now? Well, it's in the papers. In any case, she found three proteases that would discriminate, and especially the 20S proteasome, which was almost completely dependent upon oxidation before it would attack an enzyme. Well, having found this unique requirement for oxidation in order to initiate proteolytic degradation, I looked in the literature to see what might have been done in the past, and I was very pleased to find that during aging, it had been shown that... Oh, I should have mentioned before that these oxidation reactions led to the inactivation of the enzyme. So when I was surveying the literature, I found that during aging, it had

[4] Jennifer Rivett

been demonstrated that there were a number of enzymes that accumulated in catalytically less active or non-active forms. The less active forms exhibited rather unique biphasic thermal denaturation curves as compared to the wild-type enzyme, which was the normal enzyme, which was on a logarithmic scale, and it gave a linear rate of decay upon raising the temperature above the ambient temperature. So this led to the proposition that accumulation of oxidative-damaged proteins could result from a loss and capacity for proteolysis to occur during aging, and therefore you would get the damage but it wouldn't be turned over. And then we tested the hypothesis that aging had an effect on enzymes, and especially on the amount of oxidized enzyme that was present in the cell. This was possible because Rodney Levine, who then was a postdoctoral fellow in my laboratory, had discovered, along with a visiting scientist from Israel, Shmuel Shatiel, they had discovered that when glutamine synthetase was exposed to this mixed-function oxidation system, that there was a generation of carbonyl groups. Some of the amino acid residues in the protein were being oxidized to carbonyl derivatives. And I took advantage of that observation to develop a technique that would allow us to measure the carbonyl content of proteins by reaction with 2-4 di-nitrophenylhydrazine and getting rid of the excess reagent and then looking at the spectrum of the protein complex, and you could see accumulation of carbonyl groups in proteins in that manner following oxidative damage. So, taking advantage of that technology, Cindy Oliver,

who was here and has in the meantime, she went from here to Merck and then from Merck, because her husband was still in this area and she wanted to come back to this area, she's now involved in one of the biotechnology groups here in this area[5]. In any case, what she found was that when you looked at fibroblasts of individuals of different ages--these are cultured fibroblasts, but the donor age was different for the fibroblasts. And what she found was that there was an exponential rise in the amount of oxidized protein in the cell as a function of age of the donor, and that this had little to do with the number of cell divisions that went on in culture, it was really related to the donor. Based on that observation, I proposed at a symposium that I was asked to participate in, a symposium on aging (this was the first time I ever got involved in aging--I was asked to give a paper). I presented the work on oxidative damage by oxidation and presented the results of the studies on the fibroblasts in support of the concept that aging led to an accumulation of damaged proteins, namely oxidized proteins. In the meantime, soon thereafter, another group in Kentucky and Oklahoma--it was a collaboration between them[6]--demonstrated that if you looked at brain tissue in humans as a function of age--and they had ages ranging from around 30 or so up to 80--they also found that the carbonyl content of proteins in those, in the brain tissues, increased also, in a parallel manner to what was observed in the

[5] Medimmune

[6] John Carney and Robert Floyd

fibroblasts, cultured fibroblasts, which lent greater support to the concept that aging in fact was associated with an accumulation of damaged protein. Then a person came to the laboratory that... Oh, I told you that I had two postdocs from Hopkins. One was Roseman[7]. And, incidentally, this paper deals with the covalent modification of proteins by mixed-function oxidation. It was one of the important early studies that was carried out in this area. Here's one of the first in a Proceedings of the National Academy paper that we published on this. That was in 1983. But this symposium that I told you about was already three years earlier than that, and that work didn't come out until two years after the symposium was. But it was published in a book, not in... Okay, where were we? I was talking about...

Park: If we're going further, may I ask you the context of your research, the context of NIH? I mean, what you are saying is that the research, your research progressed along the line of your interests in basic research and...

Stadtman: Well, that's for sure.

Park: Yeah, and from glutamine synthetase and oxidation.

Stadtman: And suddenly you find yourself in aging.

Park: Yes. You found yourself...

Stadtman: [A natural] progression. It's based upon correlations between what you do and some other phenomena that lead you to suspect that maybe it's involved in this or that process.

[7] Jo Ellen Roseman, who was a graduate student in the NIH-Hopkins joint program.

Park: So that means that nobody asked you to find something applicable from your basic research -- say, the scientific director came to your lab or Dr. Stetten[8]. Can you find anything useful from your research? Did anybody ask you to do that, or just...

Stadtman: No. As a matter of fact, there was once... At one time in my career, I was offered a very good job in Texas, and my wife also was offered a very good job there, so we seriously considered leaving NIH and going there. And this happened at a time when there was a big movement here at NIH to get people involved more in project-oriented research.

Park: When was...

Stadtman: I don't recall the exact year now.

Park: Early '70s, during the mid-'70s?

Stadtman: It would have been in the '80s. Well, maybe it could--yeah, it could have been in the '70s, as a matter of fact[9]. I think Stetten was then the administrative director, intramural director at NIH. In any case, when he learned that I was considering leaving, he called me in to his office and said, "I understand that you're planning to leave NIH, so I'd like to know why." I told him that I sensed from the press and other signals around NIH that there's going to be some pressure put on people to concentrate more on project-oriented studies, and that from the time that I came here, I had always been allowed to work on pretty much what I wanted to work

[8] Dewitt Stetten, Jr

[9] It was ~1976, and Stetten was the Deputy Director of NIH then.

on, and that that would be true if I'd left and taken this job. And he said, "Dr. Stadtman, as long as I'm... When people ask me, is there any fundamental research going on at NIH, I always called attention to your lab as the one example of fundamental, basic research going on. And as long as I have anything to say about it, I can assure you that you will never be required to become involved in project-oriented programs." But, as happened, of course, these studies always uncovered basic principles that are pertinent maybe not only to Heart but to many other disciplines as well. That's why I think basic research is not given as much credit as it deserves in the annals of public opinion.

Park: Right, especially...

Stadtman: This is a particularly important paper on the early studies on the role of... Oh, wait. That's not the one I had in mind. That summarizes some of it, but there was another one. This was all right, but there's another one by Cindy Oliver and me. Yeah, this one. This one is really very important because it was that first paper on the... I'm sorry. This one, not this one. This one described these experiments with the fibroblasts, and I think that was a really very key and important paper, although I had already reviewed it many years before it was published in 1987. In any case, after the experiments with the brain tissue that I told you about and the fibroblast studies, I suggested to one of my postdocs[10] that she look in rats, rat liver, and see if there was any age-related correlation between

[10] Pamela Starke-Reed

protein oxidation and age, and so she got some rats from the--it's an NIH program, outside program, where they grow animals for distribution to various peoples, and they had animals of various ages. And we got a series of animals from them of different ages, and when she looked at the liver of those animals, she found a progressive increase in the level. And if you normalized for the age--in the case of rats, it's almost 30 months, something like that, 26 to 30 months is the maximum life span--if you normalized to the life span, then those results also fit the same curve that we had seen with the fibroblast culture in the brain studies that I told you about. In addition, a study carried out by Donna Garland over in the Eye Institute, she measured the carbonyl content in the eye lens of people of different ages, and her results showed the same pattern as a function of age. So we were convinced that there was this age-related accumulation of damaged enzymes. And with that method, the carbonyl procedure that we used, it became standard practice in many laboratories throughout the world, measuring protein oxidation as a reflection of oxidative stress and that sort of thing. In fact, we showed that if you exposed animals to 100 percent oxygen for, well, in the case of a rat, a rat will die after about 50, 60--55 hours is the maximum, I think, that they can tolerate. And if you take samples at different hours during the exposure, you saw an increase in the carbonyl content of the... So oxidative stress was then considered one of the possible damaging areas for protein damage, and probably for other molecules as well. But, in any case, this work then led people to look

using... Well, let me first go into another state of this. I had a postdoc come to me from Japan, and he was... I see I'm getting kind of ahead of the game here, so let me come back to that later. We got involved in looking at the oxidation of free amino acids, because I wanted to find out what the free amino acids were converted to in the course of oxidative damage. So we used the mixed-function oxidation system to study the oxidation of free amino acids. Now, we did that as a consequence of the paper that came out by Robert Floyd in which he stated that, on his study--he was studying the [free] radicals formed when amino acids were exposed to what he referred to as the Fenton system, which is iron plus hydrogen peroxide. Now, to clarify why hydrogen peroxide is involved, I should point out that these mixed-function oxidation systems that I talked about all generate hydrogen peroxide. The hydrogen peroxide was known from previous studies to be cleaved by iron and copper to give a hydroxyl radical, which is an extraordinarily reactive species, and it just will destroy anything that's [nearby], oxidize anything in the neighborhood. So, I thought, well, we can maybe learn something about amino acid metabolism by following the lead of this investigator. He had used sodium bicarbonate as the source of buffer in his system because he didn't want to have things like Tris or Hepes or things like that around because they could be misleading when you started looking at the signal, EPR[11] signals,

[11] Electron paramagnetic resonance spectroscopy (EPR), also termed electron spin resonance spectroscopy (ESR)

because of reactive oxygen species could react with those buffers and get products that would be different from the products that you get with amino acid. Which is what you really are interested in studying. So we started using pretty much the system that he had described and found that, in our hands--and we now were using, not EPR measurements, but, rather, Warburg experiments[12] where we could measure the amount of oxygen consumed or CO_2 produced manometrically and get a quantitative relationship between oxygen consumption and CO_2 production. Then, at the end of the oxidation, take the reaction mixtures and determine what the products were which were formed as a course of this oxidative process. But when we tried to change buffers--I didn't want to use the bicarbonate buffer because it's so hard to control under... You can do it, but it's kind of hard to control the pH when you have a bicarbonate buffer, and so we tried other buffers. And much to our chagrin, we found that there was no oxidation of amino acids in the absence of bicarbonate. Bicarbonate somehow is a key player in this whole process, and that, of course, was all right with the manometric techniques because you can in fact, in the Warburg apparatus, you could control pH by varying the bicarbonate level of the reaction mixture when you used 5 percent CO_2 as the atmosphere. So it was possible to adjust the pH over a wide range and maintain that pH under very controlled conditions, which allowed us to do these Warburg

[12] So called because they employed an apparatus invented by Otto Warburg that could measure the amount of a gas produced or consumed.

experiments. But, anyway, that gave us a lot of information on the mechanism of oxidation of free amino acids. And, curiously, when we got around to publish this work, I wanted to know whether anyone else had ever used the Fenton system, iron plus hydrogen peroxide, to study amino acid metabolism. So I went to the library and looked at back issues of, in Chemical Abstracts and the other sources of references for published work, and much to my chagrin, I found that in the very first issue of the *Journal of Biological Chemistry*, there was a paper by Dakin[13] describing the oxidation of amino acid by iron and hydrogen peroxide. And when I read that, I was amused that he said we adjusted the pH of our reaction mixtures by adding sodium carbonate. He was unaware of the fact that bicarbonate was required for the oxidation, but he inadvertently was adding it to neutralize his reaction mixtures, and therefore these oxidations were also carried out in the presence of bicarbonate. Kind of an amusing story. Where are we? Yeah. Here is--these are some rather important papers that deal with... One of the things which we learned from these studies was that if you use manganese instead of iron, you still got oxidation of amino acids in the Fenton system. If you replaced iron with manganese in this system, you still got oxidation. And in looking at what happened in the absence of any amino acid, we found that manganese

13 H. D. Dakin The oxidation of amino-acids with the production of substances of biological importance. J. Biol. Chem. 1: 171-176 1976. As an aside, the Journal of Biological Chemistry was founded by Christian Herter, and Dakin worked in Herter's lab. That is probably why Dakin chose to publish his work in this start-up journal. He surely had no concerns about impact factors.

would catalyze the... I'm sorry. We established that when you put hydrogen peroxide in the presence of manganese and amino acid, there was a disproportionation of the hydrogen peroxide in bicarbonate buffer. The hydrogen peroxide was, just like catalase destroys hydrogen peroxide, converts it to water plus oxygen, and that's what happened in this case. So, taking advantage of that observation, Peter Ward later carried out some studies with growing cultures, and he showed that if you added manganese and amino acid and work in bicarbonate buffer system, that you could inhibit oxidative damage to proteins and other cell killing. It was a detoxifier. So it protected cells from killing by inflammatory cells, for example, and so that observation was key to a further development on the protective role of manganese plus... And we're still doing some experiments on that, but they aren't yet in a final state. The other... This one I've already alluded to. This is the paper that deals with the gerbil brain studies where you get... Well, I guess I didn't mention it. Ischemia re-perfusion injury in gerbil brain leads to production of protein oxidation [and appearance of] carbonyl groups, and that's what this paper describes.

Okay. Now, I think we've been wandering a little bit here. In a number of these review articles, you'll see summarized some of the concepts that I was telling you about. But what became important as an outgrowth of this whole field was that protein oxidation occurred also during a number of diseases. There was an accumulation of damaged protein in... If you did

just a chemical analysis of tissue slices from humans as well as animals, you could see that when they--if you had an animal model for a certain disease, that there was an increase in the amount of protein carbonyl content in the animal, in certain tissues of the animal, associated with the disease. And as a consequence of these studies, it was clearly established that protein oxidation is involved in a whole host of diseases, especially neurological diseases, in the case of Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, in the case of diabetes--well, there's a whole list of them. They're summarized in some of these papers, where people have demonstrated that, by histochemical methods, that you got accumulation of oxidatively modified proteins during the development of the disease. So it opened up another marker that could be to examine whether or not oxidative stress was in any way involved in the progression or development of a given disease, and the evidence is pretty good...

Stadtman: [Tape begins again with] …diseases of the heart.

Park: So your research seems to give a fundamental knowledge about disease...

Stadtman: And the development of technique, in this case, which can be utilized to test a hypothesis, and it's been widely used by many people. And I've summarized that in a number of reviews in this work where I, say, in protein oxidation and aging and disease, so all of those diseases are covered in the review articles that I've written in this general field.

Park: Last year I attended a lecture by Dr. Bruce Ames about cancer and how oxidation was related to DNA damage. And I remember that he cited your

work.

Stadtman: Yeah. Well, it was... But we also have, you'll find in reading the reviews that I've written, suggested that the susceptibility to protein oxidation may be to a large extent a reflection of DNA damage, the concept being that during the process of aging or in development of a disease, oxidative stress, exposure to oxidative stress, you will cause mutations to occur. Now, most of the DNA strand breaks are repaired, but a very small fraction, maybe a hundredth of a percent or less--I don't know; it's a very, very small fraction--of the oxidative damage to DNA escapes repair and leads to mutations. And if these are random, which I suspect they are. Time, over time, aging, for example, you can accumulate a lot of genetic errors, and these genetic errors will influence what happens in the sense that this oxidative process that I talk about is so complicated, it involves all of the x-ray, various rays from the atmosphere that you're exposed to, ozone in the atmosphere. It derives... Hydrogen peroxide is produced as a byproduct through the generation of superoxide anion. It's produced in the course of normal metabolism, oxidative metabolism. It's been shown that about 1 to 2 percent of all of the oxygen that is consumed in the normal respiratory chain leads to bleed-off of superoxide anion inadvertently. And that in turn is converted to hydrogen peroxide, which can give rise to hydroxyl radicals by the methods that I've alluded to. And then in a recent summary, I pointed out, all of the sources of reactive oxygen species that we're constantly exposed to--and there's a whole host

of different mechanisms for generating reactive oxygen, one of them being the cytochrome P450 systems[14]. Cytochrome P450 utilizes NADPH as an electron donor, but it requires oxygen and it, in the absence of its substrate, you get an accumulation of reduced cytochrome P450s, which then undergo auto-oxidation, and that leads to the generation of free radicals. And, in fact, we showed it in our studies earlier that if you expose proteins to any one of several different P450 systems, cytochrome P450 systems, and an appropriate electron donor, that you would get oxidation of the protein. So that's another source of reactive oxygen species in the course of normal metabolic process. There are several hundred P450s in the cell, and most of them probably have this capacity to generate reactive oxygen species under certain conditions.

In any case, what I'm trying to get at is that if you had a genetic alteration that led to an increase or modification of enzymes that are normally involved in antioxidant activities, protecting against damage, or if you had a mutation which would lead to the generation of more reactive oxygen species, then that would lead to an increase in the accumulation of oxidized protein.

And then we discovered in the course of these investigations that not all enzymes, when they're oxidized, are degraded by the systems that recognize oxidized protein, these proteases. But, in fact, they are

[14] These were the first mixed-function oxidation systems described – by Udenfriend and colleagues.

inhibitors of the protease, and more extensive study has shown, in some cases, this is due to crosslinking reactions. You get protein-protein crosslinks, and those crosslinked proteins, as a consequence of oxidative function, cannot be proteolyzed by the normal proteases but in fact will inhibit the ability of the proteases to catalyze the degradation of other oxidized proteins. So there's a situation here where oxidation doesn't always lead to, depending upon the protein which is oxidized--and that could be governed in part by genetic functions--you might generate a crosslinker of the protein, which could be very serious in inhibiting the ability to get rid of those damaged proteins so they don't accumulate in the cell. Well, I was looking for the work of this Japanese co-worker[15] that I had, and I want to get to that because...

Park: [question garbled?]

Stadtman: No. That's an Italian postdoc.

Park: I see.

Stadtman: Here we come. Uchida [learned from Hermann Estebauer's group in Austria] that when lipids are oxidized, they also give rise, by mixed-function oxidation systems or other oxidative processes, they generate alpha-beta unsaturated aldehydes as one of the products, one of the major products that are formed, and 4-hydroxynonenal is another product which is really prominently generated. And they demonstrated that these alpha-beta unsaturated aldehydes could react with cysteine residues in proteins

[15] Koji Uchida, a post-doctoral fellow with Earl.

very rapidly, or with cysteine residues in glutathione free cysteine. Any sulfhydryl compound would react with these oxidized products to form a thiol ether which would have a reactive carbonyl group, an aldehyde function associated with it. I wanted to know whether or not these aldehydes that were formed from lipids could contribute to protein modification by virtue of the fact that the aldehyde, lipid aldehyde derivative might then react with the lysine residue on a protein to form a Schiff base. That in turn could be converted to something else which would lead to a modified protein. So I had Uchida develop procedures where he could measure the amount of thiol ester--not thiol ester, yeah. Not thiol ester. Oh, what is it[16]? It's the primary derivative. You know, I'm losing my mind. As I get older, I can't remember things as fast. What did I say before when I talked about when cysteine residues react with these? You get thiol ethers. And these thiol ethers, I wanted to know whether or not he could develop a technique which would allow him to measure the level of thiol ethers in proteins, and he did. He worked out a very nice procedure which involved reaction with borohydride to stabilize the carbonyl group, and then you could degrade the complex and measure the amount of thiol ether that was formed. It was found that that accounted for only a small fraction of the carbonyl group that we measured in oxidized proteins, but it did contribute. In the course of those studies, he found that not only would these alpha-beta unsaturated

[16] Thiol ether

aldehydes react to thiol groups of proteins, but they react with lysine residues of proteins directly and also with histidine residues of proteins. He developed procedures for measuring the amounts of those complexes and also developed some immunochemical techniques that would enable you to measure the presence of some of these complexes, the main one being hydroxynonenal, which is the alpha-beta unsaturated product that you get from many lipids that you can examine. He found that he could get the thiol ether out and quantitate it, but he also showed that the histidine residues and, in one of these papers, lysine residues that were also modified. And this has led now to the use of antibodies of this type to look also whether or not these kind of complexes contribute to diseases. And there's very good evidence for its involvement in Alzheimer's disease, Parkinson's disease, in some of the heart diseases. I'm trying to think of the name of the... Let me look here…. atherosclerosis is what I was trying to think of. It's involved in atherosclerosis as well, and in a number of diseases which we have alluded to in these review articles that I have written.

But, anyway, these are important papers from that point of view because they deal with the oxidation of proteins. And, among other things, [Luke I.] Sweda, who was a postdoc in my lab, showed that when you oxidize glucose-6-phosphate dehydrogenase in the presence of hydroxynonenal, you get adducts which crosslink other proteins, and these derivatives are very resistant to proteolysis and very potent inhibitors of the ability for

proteolysis to occur. So here's the immunochemical detection that was developed by Uchida and used by Sweda and other people in the lab. And those techniques are now used widely in these diseases that I was telling you about. You'll see, for example, there'll be--here's one on carcinogenesis by iron-nitrilotriacetic acid. This is one of the common carcinogens that people have used to study. This is a case where you can get these cross-linked derivatives that are very resistant to oxidative degradation by proteases. Here's some evidence for the role in atherosclerosis, and as you can see how these studies evolved, you get involved in studying oxidative damage, and these adducts could also give you carbonyl derivatives, you see. So if you--you want to know how much of the carbonyl that you measure when you measure by the 2-4 dinitrophenylhydrazine technique is due to these reactions.

Then it was discovered by other workers, especially by John Baynes and the group in South Carolina, who have shown that glycation can also lead to the generation of carbonyl derivatives in protein, and glycation is something that occurs when sugars that have a reactive carbonyl or aldehyde function, fructose or glucose, for example, when those react with lysine residues, what is referred to as the Maillard reaction[17], you get a rearrangement of the complex that is formed to form a more stable

[17] As Earl mentioned at the very beginning of the interview, his war work included studies on the Maillard reaction as it related to the browning of apricots.

derivative, which is subsequently metabolized in the presence of metal ions and oxygen to give, in some cases, carbonyl products, which are also included. When you measure carbonyl groups, you get... You're measuring not only direct oxidation of amino acid, but you're measuring the amount that is produced as a consequence of glycation reactions and of these fatty acid oxidation products, which can form derivatives with proteins. So it's a very broad marker, if you will. It includes something that can mark all of those different activities, give a gross idea of what oxidative damage has occurred. Well, how are we doing?

Park: Fine. We can stop here or continue if you want.

Stadtman: All right. Let's stop here, and I'll take up a new project next time.

Park: Right.

Stadtman: But, anyway, as you can see, the discovery of oxidative damage to proteins has given a lot of clues. Oh, I should tell you one story about the oxidative damage. I told you of some experiments that we did where we oxidized, took animals of different ages and rats of different ages and showed that, in the liver of those animals, there was an increase in carbonyl as a function of age. Well, about five to 10 years later--I forget just how many years, but many years past--we had occasion to repeat those experiments, and we got some new animals in. And much to our chagrin, during aging, there was very little carbonyl group formed. There was some, but it was nowhere near the amount that we had seen in the earlier batch of animals. Yet these animals came from the same source

and had presumably been dieted and everything the same way. At least they claimed that they had not changed their dietary procedures. So we were really up against trying to explain this, and yet it was clear. And I thought at first that maybe the collaborator, the postdoc that had done the earlier studies, maybe had blundered somewhere. But, fortunately, we had in our -80° [freezer] samples from the earlier experiment, so we took those samples out and now measured them again using even a more refined carbonyl assay than we'd used earlier, and we found exactly the same pattern that she had found in the original experiments, this age-dependent increase in oxidative damage. So the question is, what went wrong? And then we learned that these rats are now living about 30 percent longer than the earlier batch. So my guess is that, in the course of breeding, they have developed strains of rats which have more resistance to oxidative stress or, for one reason or another, have mutated and are different from the original strain, which would account for the fact that, in the case of the liver studies, there had been two other groups that had failed to see a substantial increase in carbonyl content as a function of aging. But in our case, we know it's not the method that's wrong, it's the animals that have changed. And you might ask, why would that be important? But it's important because, for example, if you take animals and treat them with low doses of reactive oxygen, which are toxic but do not kill the animal for a period of time, and then take them off the exposure to the reactive oxygen for a while, and then expose them again, they can tolerate really

very high levels of the oxidant situation, oxidative stress that you expose them to[18]. So it's conceivable that you can develop strains which are more resistant to oxidative damage, and that may reflect, of course, genetic mutation. It could be a question of selection among populations, because as you breed over time, we'll always breed the most vigorous animals and we could end up with a strain which is different from what it was 10 years before. So that's our explanation for that. And it's known also, for example, that if you expose rats to dietary restriction, that they will live 30 percent or even two times longer than if they are given a diet where they can eat as much as they want. And if you measure the carbonyl content in the diet-restricted animals, it's more than the carbonyl content of the ad libitum fed animals. So it's clear that you can change the carbonyl content of proteins by manipulating the animals in such a way as to favor this or that situation.

The same sort of thing has been found with flies, house flies. If you expose house flies to x-irradiation for a limited amount of time and then, but not lethal, take them off of it for a while so that they can recover well, and then expose them to the same dose or a higher dose, they can tolerate it without any problem. So the history of the animal is very important in this process as far as oxidative damage and resistance to it. Part of it, I'm sure, is genetic. Part of it is a consequence of mutation. But, in any case,

[18] The general phenomenon is termed "hormesis".

it's very clear that the way in which animals are grown can influence what you find, and also how long they grow, cultivated on the same diet over a period of time. Whis out to some people--I discussed it at one of the meetings I attended--and the person in Japan, who had failed to observe an increase in carbonyl content in animals, although he had shown increases in other systems, so it wasn't a question of the carbonyl assay that was in doubt, it was just that he couldn't confirm what our original observation was. When I told him this story, he came up to me afterwards and he said, "Gee, I was delighted to hear that." He said, "I've always been puzzled by why we couldn't repeat your experiment," which was published much earlier.

There are many factors that are involved, and all this does is show how complicated the systems are. But it may lead to some important developments way beyond what we imagine at the moment. I think the fact that exposure of animals to a sub-lethal dose of reactive oxygen increases their tolerance to more toxic doses if they're allowed a breather in between. And that, I think, is a very important observation in this whole field, that maybe it's not so good to deprive everybody of exposure to a little bit of oxidative stress. If you eliminate all oxidative stress, you might find that people are more susceptible to oxidative stress when it comes.

Park: Right. Radiation is the same.

Stadtman: Exactly, the same thing.

Park: It's a dangerous thing, but we can use …

Stadtman: If you expose animals to a little radiation and then give them a brief time to recover, and then expose them to a higher dose, they can tolerate the higher dose.

Park: That's very interesting.

Stadtman: A good example of that, actually, is in the case of the 100 percent oxygen. If you take a rat and expose it to 80 percent oxygen, it will survive--not too well, but it'll survive. And then, after a recovery period, if you put it in 100 percent oxygen, instead of dying after 48 hours, it lives perfectly normally in 100 percent oxygen. So there's a difference between 80 percent and 100 percent oxygen in the severity of the damage that occurs, and apparently it induces enzymes that allow the animal to now tolerate 100 percent oxygen without any problem.

Park: Sounds like a vaccination.

Stadtman: Exactly.

Park: Or developed immunity to oxidative...

Stadtman: But the whole... I mean, oxidative stress, there are so many forms of it. You have--too much exercise gives you oxidative stress. In fact, here's a good example. If you take flies and put them in a small chamber where they can't really fly because there's no room for them to fly, or if you pull the wings off of the fly so that it can't fly, it'll live twice as long as the flies that can just fly at will.

Park: Amazing!

Stadtman: If you measure the carbonyl content, you find that the fliers have a lot more carbonyl content in their proteins than those that couldn't fly. So, again, there's this correlation between life span and the amount of oxidative damage that occurs.

Park: Did you coin the term oxidative stress, or it was there? Did you make the term?

Stadtman: I wouldn't say that. No, I didn't coin the term oxidative stress. I think that's something that has been alluded to before, especially by the people working in DNA modifications.

The history of oxidative damage goes back before the protein story. It really began when Denham Harmon postulated the so-called oxidative free-radical theory of aging, and he proposed that free radical generated during metabolism or exposure to ionizing radiations or what have you could lead to damage, and people who were investigating this area selected DNA and lipid primarily. They never looked at proteins. So I think we were the first to use proteins as a measure of oxidative stress, and it's now being used by many, many people. In fact, if you look at the citations to carbonyl groups in proteins, it's been going up exponentially over the last several years. I think last year alone there were something like 300 citations to use of the carbonyl as a marker of oxidative stress.

Park: Your paper must be one of the most cited papers.

Stadtman: It's very often referred to, yeah, especially some of the review articles that I've written. I wrote a review in *Science* and one in *The Journal of Biological Chemistry*, and they're very, very often referred to as a source of information on protein oxidation. But, in any case, I think we were primarily the group that called attention to the importance of protein oxidation. Bruce Ames has concentrated largely on DNA, and he made some rather amazing observations. In one review, he said that calculations based on the amount of 8-hydroxy guanine that's formed and one of the other oxidation products of DNA, that there are on the average about 10,000 strand breaks per cell per day. But the efficiency of the repair process is so good that it's only a very, very tiny fraction that escapes repair, and only special circumstances can increase the amount. But that's an amazing figure you have. That gives you an idea of how much oxidative damage is going on. If 10,000 DNA strands break per cell per day as a consequence of oxidizing residues on the DNA, that's amazing. But that gets repaired, and the difficulty with the protein oxidation is that there are no repair mechanisms. Except as I'll discuss later, methionine oxidation and cysteine oxidation, there's no really repair mechanism involved. So the only way you can get rid of the damaged protein by oxidation is to degrade it and eliminate the abnormal components and use the rest of them for resynthesis.

Park: Very chemical ways.

Stadtman: Yeah. So it's a very serious thing, protein oxidation, in the sense that you

get an accumulation that is much greater than the accumulation that you see in other types. But lipid oxidation is very good, and I'll come to that more than what I've already done. We certainly are aware of the nitric oxide/peroxynitrite story. That's one that we were involved in also earlier, which is now receiving enormous attention. But people were in that field long before we got into it. But what our current stuff is dealing mostly with is methionine oxidation because we think this is going to turn out to be one of the more important, and, unfortunately, that doesn't lead to carbonyl groups, so...

Park: Well, we can stop here for today.