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Inositol, Glycogen, Insulin, and Six Nobelists

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My reflections cover my scientific work over a sixty-year period. At the University of Michigan, I was initially interested in embryology. In medical school in the army, I became entranced with biochemistry and DeWitt Stetten, who introduced me to Carl Cori. Carl along with Gerty Cori and Earl Sutherland became my scientific mentors. After medical school and a nine-month medical internship, I re-entered the army and was assigned to Edgewood Arsenal in Maryland, where I established the NAD requirement for brain pyruvate dehydrogenase to oxidize pyruvate and published my first *Journal of Biological Chemistry* (JBC) paper.

At the University of Illinois, I obtained an M.S. degree in chemistry and was influenced by Will Rose and Herb Carter. At Washington University in St. Louis, I worked with Gerty and Carl Cori on glycogen metabolism and obtained my Ph.D. degree. I also worked with Bill Daughaday on the increased urinary excretion of *myo*-inositol in diabetic subjects and in rats preloaded with glucose. I was offered an instructor's appointment in biochemistry at Washington University, but I decided to accept an assistant professor's appointment in the Division of Biochemistry of the Chemistry Department at the University of Illinois. I spent four years at Illinois working on intestinal starch digestion with particular reference to glycosidases hydrolyzing the α1,6-branch point linkages.

I was recruited by Earl Sutherland to join his Department of Pharmacology as an associate professor at Western Reserve University in Cleveland. Here, Carlos Villar-Palasi, my first postdoctoral fellow, and I began our insulin studies, which led to the discovery of two covalent forms of glycogen synthase controlled by insulin and epinephrine. These continued at the University of Minnesota, where I held an endowed chair in biochemistry. Here, with students and postdoctoral fellows, we further defined the two forms of glycogen synthase and worked on the possible role of cAMP in insulin action.

Moving to the University of Virginia as Chair of Pharmacology, I recruited two future Nobelists, Al Gilman and Ferid (Fred) Murad, with Ted Rall, their past mentor for their Ph.D. degrees, as well as other faculty who would become departmental chairs. Our early studies on glycogen and inositol became united with studies on insulin action. We isolated a novel inositol glycan pseudo-disaccharide insulin second messenger containing a rare inositol, D-*chiro*-inositol (DCI). Its structure was determined as galactosamine β1,4-pinitol, where pinitol is the 3-*O*-methyl ether of DCI. It was chemically synthesized. We and others found that insulin resistance in several human diseases is related to an inositol imbalance consisting of a deficit of *chiro*-inositol and an excess of *myo*-inositol, an unfolding story now under way involving the *myo*-inositol to *chiro*-inositol epimerase.

I am blessed to have had such superlative scientific mentors, a productive group of students and postdoctoral fellows, a loving wife of sixty-five years, three remarkable sons, and eight wonderful grandchildren. I am working full time on insulin, which has entranced me for a long time.

Undergraduate Work at Michigan

During my college years in Ann Arbor, I lived in a "co-op" house, where we all shared the house duties. It cost $5 a week for room and board. In addition, I had a job as an orderly at the university hospital, where, among other duties, I cleaned bedpans. I was a premed major and graduated with

a B.S. degree in zoology in 1942. I was interested in embryology and, as a senior, was assigned a research lab bench adjacent to the graduate students. I worked on amphibian limb regeneration. I am tremendously grateful to the University of Michigan for this privilege as an undergraduate and years later for the honor of a Sesquicentennial Award for Distinguished Alumni.

Medical School and Army Stint

In medical school at Columbia University College of Physicians and Surgeons in New York City during World War II, I was influenced by DeWitt "Hans" Stetten, who inspired me to study biochemistry. He arranged for me to meet with Carl Cori, who accepted me into his graduate program at Washington University in St. Louis. However, the offer was conditional and required that I first successfully complete a master's degree in chemistry at the University of Illinois.

After earning an M.D. degree in 1945, I completed a nine-month internship at the University of Chicago. I was then inducted into the Army Medical Corps as a first lieutenant and was sent to Fort Sam Houston in San Antonio, Texas, for the medical officer training course. During a morning reveille, the announcement was made, "Anyone interested in research take two steps forward." I was one of three who volunteered and was assigned to the Chemical Warfare Center at Edgewood Arsenal in Maryland (Fig. 1).

At Edgewood, I published my first JBC paper defining the NAD^+ requirement for pyruvate dehydrogenase (PDH) oxidation in brain (1). PDH was a sensitive enzyme target for chemical warfare agents, and I convinced the brass (commanding officers) that it was important to know the cofactor requirements before applying the toxic agents. I was able to purchase NAD^+, but I needed to check NADP, which was difficult to obtain. I contacted Severo Ochoa, who was then Professor and Chair of Pharmacology at New York University. He graciously weighed out a few milligrams for me on his own balance. It was inactive, and NAD^+ was the active nucleotide. Al Lehninger, whom I had known in Chicago when he was a young faculty member there, liked and quoted my paper.

I married and lived on the post with my new wife, Fran, in an efficiency apartment with an icebox that used ice as coolant. A dealer supplied cakes of ice several times a week in the morning, rousing us out of bed. I completed my two-year stint at Edgewood, which included some time at the Dugway Proving Ground in Tooelle, Utah, where live animal tests with chemical agents were conducted.

FIGURE 1. **First lieutenant Joe and Fran Larner with neighbor's child (1947).**

Graduate School in Chemistry at Illinois

We then moved to Champaign-Urbana, where I began graduate studies in chemistry. There, we had an open attic bedroom in a little bungalow. The owners (Ma and Pa Shanks) lived on the first floor with the one bathroom, which we all shared. At Illinois, I was very impressed by the inspiring lectures of Will Rose on metabolism and by Herb Carter. In a course entitled "Vitamins and Hormones," Herb challenged us to come up with a mechanism to explain the role of inositol deficiency in producing fatty liver and alopecia. This had been the discovery of the noted scientist at The Rockefeller Institute (now The Rockefeller University), D. W. Woolley (2). In the German literature, I found a reference to the increased inositol excretion in urine in diabetic subjects, which I thought might be a good place to start. My hypothesis was that the increased glucose in blood and tissues in diabetes might lead to increased formation of inositol in the body and increased excretion in urine.

Graduate School in St. Louis

In 1949, I completed my two-year M.S. degree in chemistry at the University of Illinois, where we formed many

warm friendships among the students and faculty. In an organic chemistry lab, I met Bob Haynes, an M.D. also taking a degree in chemistry, whom I would later hire as my first faculty appointment at the University of Virginia. We moved our belongings by truck to St. Louis, where we rented our first apartment.

Carl Cori assigned me to his wife, Gerty, and her laboratory. It was just across the hall from Earl Sutherland and his laboratory. Earl and I immediately became very good friends. Gerty's lab was equipped with pipettes, a balance, a Klett-Summerson colorimeter, a centrifuge with a wall-mounted rheostat control, and a cold room. Of interest, Bill Summerson, the co-inventor of the colorimeter, had left academia, joined the army, and had been my boss and supervisor at Edgewood Arsenal. The Cori lab benchtops were covered with a black tarry composition material. There was a small desk for me in the lab and a separate office with a cot for Gerty, who required rest and medical care because she was ill. She was still strong enough to supervise my pipetting technique and finally accepted my skill only when I produced a glucose standard curve that was a straight line and went through the origin. She taught me to crystallize rabbit muscle phosphorylase, and that became my first project. Across the hall, Earl taught me how to handle rats and to crystallize glucose 1-phosphate potassium salt from starch digested with potato phosphorylase in the presence of inorganic phosphate.

Debranching Enzyme

My first project was to find the mechanism by which crystalline muscle phosphorylase was able to completely digest glycogen in contrast with crude potato phosphorylase, which stopped at the outermost branch points, the α1,6-linkages, distinguished from the main chain α1,4-linkages. Shlomo Hestrin, a postdoctoral fellow from Israel who preceded me, claimed that crystalline muscle phosphorylase was contaminated with an enzyme that cleaved the α1,6-linkages and allowed complete digestion of glycogen. On the other hand, crude potato phosphorylase digested glycogen only to the outer tier of branch points and stopped. When Gerty returned from Stockholm with her Nobel Prize and heard the results from Shlomo, she was furious. How could a crystalline enzyme that led to the Nobel Prize be impure? After she had calmed down a little bit, she proposed that I find out what was the contaminating enzyme.

I set out to recrystallize muscle phosphorylase eight to ten times and save the supernatants from the crystals to identify the contaminating enzyme. One Saturday morning when I was recrystallizing phosphorylase, some crystals spilled on the benchtop tarry surface. Gerty watched as I sucked up the crystals with a pipette and added them to the main batch. She smiled and approved what I had done. I pooled and concentrated the supernatants as a source of the contaminating enzyme. I isolated the product of the glycogen (called a limit dextrin) remaining following digestion with potato phosphorylase or with the highly purified, recrystallized muscle phosphorylase. I treated the limit dextrin with the concentrated supernatant enzyme and looked for the product with paper chromatography, thinking, like Gerty, that it would likely be phosphorylated glucose. To our surprise, it was free glucose. This was also the product of breakdown of the α1,6-linked glucose disaccharide isomaltose, which had just been made available from Edna Montgomery, a scientist at the Northern Regional Research Lab in Peoria, Illinois (Fig. 2). Gerty was very excited; she ran down the hall to Carl's office shouting "Carly, Carly, its free glucose." Later, I found out why she had been so excited. It turned out, as Gerty told me, that years before they had completely digested glycogen with a phosphorylase and had found 90% glucose phosphate and 10% free glucose. They had assumed that the free glucose came from nonspecific hydrolysis of the glucose phosphate. Now, they knew that the free glucose came specifically from hydrolysis of the branch points (3).

Branching Enzyme and Glycogen Structure

One evening, while lying on the grass in an open-air night concert in the park in St. Louis, I had the idea as to how to measure the branching enzyme. I would build up the outer chains of the limit dextrin with [^{14}C]Glc-1-P and phosphorylase to the known average size and then treat with branching enzyme, isolate the product, and identify the newly synthesized branch point as labeled free glucose using the debranching enzyme. I asked Earl for a name for the new debranching enzyme (Fig. 3). He immediately responded, "How about amylo-1,6-glucosidase?" I said "Great," and the name was adopted. In the lab, the jargon name was "glix." The branching enzyme work resulted in a paper demonstrating that an outer chain size of six to eleven glucose residues was optimal for the branching enzyme, then named amylo-1,4→1,6-transglucosidase, with no short lab jargon name (4).

I next went on to determine the structure of glycogen. Three structures had been proposed in the literature. Carl thought that I should do serial degradations first with recrystallized muscle phosphorylase, measure the glucose phosphate, isolate the limit dextrin, treat with glix, measure the free glucose, and repeat as many times as necessary. It turned out that three cycles were all that were necessary. We now know that glycogen prepared by the

FIGURE 2. **Gerty (*1*) and Carl (*2*) Cori, Edna Montgomery (*4*), and Joe Larner (*3*) at the Starch Round Table (September 10, 1952, Skytop, Pennsylvania).**

FIGURE 3. **Earl Sutherland hard at work (circa 1960).**

old Pflüger alkaline digestion method is degraded, so what we determined was a partial structure. Carl took my entire data home to consult with Mildred Cohn's husband, the mathematician and physicist Henry Primakoff. The next morning, he came in, a bit ashamed to say that the results were simple, ~50% branch points in the first tier, ~25% in the second tier, and ~12.5% in the third tier. Glycogen was a tree-like multibranched structure (5).

On the basis of my work on the glycogen debranching enzyme, the branching enzyme, and the structure of glycogen, all of which had been published, plus an oral exam by the entire biochemistry faculty that lasted all morning, plus a German language exam that had to be repeated, I was awarded a Ph.D. degree in 1951.

An Amusing Incident

After the glycogen structure work was completed, Carl came up to me and said, "Joe, I have a much simpler way to do the glycogen structure. Let's go to the Beckman DU spectrophotometer [which was housed those days in a special temperature-controlled room] and do the structure by UV analysis. First, we'll treat glycogen with recrystallized muscle phosphorylase and detect the Glc-6-P, converted from the Glc-1-P by phosphoglucomutase, then oxidized by Zwischenferment [glucose-6-phosphate dehydrogenase], and determine NADP reduction to NADPH by UV. Next, we treat with glix and measure the free glucose by phosphorylation to Glc-6-P with hexokinase and oxidation by Zwischenferment and reduction as before. Thus, we will do the whole thing very fast by UV." Needless to say, it did not work because the two degradations got all mixed up with each other. In addition, there was an annoying fly buzzing around, which bothered both of us. After a while, I cupped my hand, made a sweeping stab, and caught the fly on the first try. Carl looked at me in amazement and said something like, "Joe, that was fantastic." He was very impressed with my skill in fly catching. We were not impressed with the results.

Glycogen Storage Disease

With my medical background, I knew about glycogen storage disease, von Gierke's disease. Following the work on the debranching enzyme, I proposed to Gerty that, in von Gierke's disease, glycogen would accumulate as limit

dextrin if there were a defect in the debranching enzyme. This would be an abnormal glycogen structure. Gerty thought otherwise. On the basis of previous work, she felt that von Gierke's disease was a defect in the glucose-6-phosphatase. I said that if her idea was correct, the glycogen structure would be normal; if my idea was right, the structure would be abnormal, a limit dextrin. We made a bet, as we often did in the laboratory. She said, "I have a set of glycogen samples, which I isolated from cases of von Gierke's disease, in the cabinet right behind you. Test them with iodine to see if they give a normal or abnormal iodine color." I picked a sample and added iodine, and instead of the pale brown color, which I expected, it was bright blue, indicating not stubs but long outer chains, more like starch. We both were aghast and stared at the test tube. By chance, the sample was from Dorothy Anderson, my pediatrics professor at Columbia University who lectured on von Gierke's disease.

I rationalized that the child had been well fed; therefore, the outer chains reflected the nutritional state, and there still might be a debranching enzyme defect despite the long outer chains. Seeing the blue color, Gerty got very excited, realizing that the abnormal glycogen structure meant a molecular disease. It turned out to be a branching enzyme deficiency. With time, it turned out we both were right: von Gierke's, then thought to be one disease, was a multiple set of enzyme deficiencies. Gerty was able to document a lack of glucose-6-phosphatase in liver, a defect in debranching enzyme, and a lack of branching enzyme. She took me off the project even though I had unpublished data on the lack of debranching enzyme in a specimen from St. Louis. I was very upset when she would not let me publish my data and told me to move to another lab and work on lipids in von Gierke's disease. She went on to win a major award for her work, well deserved. She left a $100 check, part of her award in my mailbox, which I initially refused, but she insisted. It was then that I decided it was time to leave St. Louis. Years later, after Gerty died and Carl remarried, we had him come to the University of Virginia for a Diabetes Center affair. After a few drinks at our home, Carl put his arm around me and said, "Gerty was a little hard to get along with." It was his way of apologizing.

Inositol Studies with Bill Daughaday

In St. Louis, I also worked nights and weekends on a separate project, the increased urinary excretion of inositol in diabetes. My collaborator was Bill Daughaday in the Department of Medicine. My wife, Fran, was his secretary. We found that the increased inositol excretion in urine in rats and humans was not because of overproduction, as I originally thought, but because of a renal tubular competition with glucose for reabsorption (6, 7). The positions of the hydroxyls are similar in glucose and *myo*-inositol. H. O. L. Fischer, the son of Emil Fischer, had predicted that *myo*-inositol was formed from glucose by 1,6-carbon bond synthesis. Bill and I demonstrated that this was in fact the case (8). H. O. L. Fischer was very pleased and cited our work.

Back to Illinois

I was offered several jobs but decided to move back to the Chemistry Department at the University of Illinois. Will Rose, head of Biochemistry, offered me an assistant professor position and a university rental one-story home. We moved with our first son, Andrew, just born in St. Louis. We spent four years on the faculty at Illinois, where I taught biochemistry and worked on intestinal glycosidases, particularly acting on α1,6-linkages in starch (9, 10). With students, we prepared ^{14}C-labeled *myo*-inositol photosynthetically and demonstrated its metabolism *in vivo*, leading to $^{14}CO_2$, ^{14}C-lipid, and [^{14}C]glycogen (11). We also isolated inositol dehydrogenase from *Aerobacter aerogenes*, which later became a commercial product (12). At Illinois, I usually taught about 150 hours of lecture a year, two semesters, and the summer session, as well as lab sessions. This did not leave much time for research, and I felt unsatisfied. At about this time, I received a letter from Earl Sutherland. He had moved from St. Louis to Cleveland, where he was head of pharmacology at Western Reserve University. In his letter, he wrote that he was looking for new faculty to join his department and asked for any suggestions. In the last paragraph of the letter, he casually asked whether I might be interested. I visited Cleveland and met Bob Haynes, a faculty member whom I had known as a fellow graduate student at the University of Illinois. Bob had worked previously with Earl as a medical student in St. Louis at Washington University on the initial discovery of glucagon, then called the hyperglycemic factor. I also met Ted Rall, another faculty member. I immediately accepted the job as an associate professor of pharmacology. The teaching load dropped from 150 hours to fifteen to twenty hours: more time for research. By this time, we had two more sons, both born in Urbana, and the Larner family of five moved to a home in Cleveland Heights.

Cleveland: Insulin and Glycogen

My first postdoctoral fellow, Carlos Villar-Palasi from Spain, joined me in Cleveland at Western Reserve University (Fig. 4). On Earl's advice, we decided to look at insulin-stimulated glycogen synthesis, focusing on the then-known intermediates, Glc-6-P and Glc-1-P, to determine

FIGURE 4. **Western Reserve University Department of Pharmacology Party (circa 1960).** Shown are Earl Sutherland (*1*), Bob Haynes (*2*), Carlos Villar-Palasi (*3*), Fran Larner (*4*), Fred Murad (*5*), and Dan Friedman (*6*).

a possible site of enzyme control. Earl had followed this protocol to find phosphorylase activation as the site of epinephrine action. We set up our incubator on a plywood panel in the medical student lab because our lab was not ready. We used rat diaphragms incubated *in vitro* with glucose and insulin, and we were off and running. We soon found that, in a short time of incubation (10 min) in response to insulin, glycogen and tissue Glc-6-P were elevated but that Glc-1-P was not (13). Therefore, we reasoned that there was a push from the outside to raise Glc-6-P but a pull on the inside to increase glycogen that prevented the expected rise of Glc-1-P, a crossover point. Phosphorylase was the possible biosynthetic enzyme, but our data on the ratio of inorganic phosphate to Glc-1-P argued against phosphorylase. The phosphorylase equilibrium ratio of P_i to Glc-1-P is about 3:1. Our measured ratio under conditions of glycogen synthesis was more like about 300:1 (13). This would support glycogen degradation by phosphorylase but not synthesis.

Insulin and Glycogen Synthase

At about this time, Luis Leloir announced the discovery of UDP-glucose (UDPG) and the enzyme glycogen synthase (GS). Carlos and I immediately set up the GS assay and demonstrated its presence in tissue extracts. We also demonstrated that UDPG pyrophosphorylase, found previously only in yeast, was also in tissues. We proposed the presence of a glycogen cycle with UDPG pyrophosphorylase generating UDPG and inorganic pyrophosphate, GS as the synthetic arm, and phosphorylase with inorganic phosphate as the degradative arm (14, 15). Arthur Kornberg was pleased with our scheme because it fit his idea of

FIGURE 5. **Carl Cori on eightieth birthday Festschrift in St. Louis (October 1976).**

pyrophosphate being involved in biosynthetic reactions, but Carl Cori was very upset because phosphorylase was proposed to catalyze degradation and not synthesis. Carl finally became convinced when it became clear that, in McArdle's glycogen storage disease, there was an absence of phosphorylase in muscle but an abundance of glycogen and the presence of GS (Fig. 5) (16, 17).

Carlos and I had been told via the grapevine that GS was activated *in vitro* by Glc-6-P. We checked it out on our crude muscle preparations of GS and found that it was true. We then hypothesized that Glc-6-P could be the activating factor in insulin-stimulated glycogen synthesis, as we had shown that Glc-6-P was increased in diaphragms with insulin action. We argued that GS should be activated by insulin and that, upon removal of Glc-6-P by dialysis,

the activation of GS would be lost. We found that, upon incubation of diaphragms with insulin even in the absence of glucose, GS was activated over controls. We had to run ten to fifteen experiments to be sure. The activation was rather small; however, to our complete surprise, the activation remained upon dialysis or after ammonium sulfate precipitation and reconstitution of the enzyme (18). Thus, it was a stable activation. With Jim Craig, we ran the diaphragm experiment with epinephrine in place of insulin (19). To our delight, we saw the opposite effect, an inactivation of GS. This reciprocal activation of GS by insulin and inactivation by epinephrine made good sense physiologically.

Readily aware of the concept of two interconvertible forms of phosphorylase, we hypothesized two forms of GS and set out to determine whether they existed. We proceeded to purify GS, testing the various fractions with and without added Glc-6-P. Initially with Dan Friedman, a graduate student, and then with a number of students and postdoctoral fellows, we found that ATP inhibited GS and made it sensitive to activation by Glc-6-P (20). Conversely, with Mg^{2+} and an SH reagent, GS was activated and lost its sensitivity to Glc-6-P. We termed the two forms D (for dependent on Glc-6-P) and I (for independent). We used [^{32}P]ATP to show that the conversion of the I form to the D form occurred with ^{32}P incorporation into the enzyme (20).

We purified and kinetically characterized the two forms of GS from liver, muscle, and heart (20–24). We demonstrated that there was a kinase stimulated by cAMP, kindly supplied by Earl, which carried out the GS phosphorylation, which we termed transferase I kinase (25). It was later renamed cAMP kinase by Krebs, Fischer, and co-workers (26). Conversely, a Mg^{2+}-stimulated phosphatase converted the D form by dephosphorylation to the I form, later identified in Japan as phosphatase 2C (27). This was later cloned at the University of Virginia by Shinri Tamura, Kevin Lynch, and myself (28). We determined that there were six phosphorylation sites in GS (29). Peter Roach prepared a set of GSs with increasing degrees of phosphorylation and demonstrated graded kinetic effects, which he published in JBC (30, 31). At the National Institutes of Health (NIH), Earl Stadtman told me how much he liked this two-part paper, but Krebs and Fischer were upset because it did not fit the phosphorylase monophosphorylation model. They claimed that the GS enzyme was an oligomer of six low molecular weight monophosphorylated subunits. We had already shown that GS had a molecular weight of about 85,000, which they were loath to accept but finally did.

Minnesota Years: A Book

Following a year's sabbatical in Cambridge, England, with Fred Sanger, I was offered the Boyer Professorship in Biochemistry at the University of Minnesota. I was told that Paul recommended me for this position. Earl Sutherland was recruited to Vanderbilt University in Nashville by Rollo Park, a former colleague at Washington University in St. Louis, now head of physiology at Vanderbilt. Earl was tired of being a chair. He also left Western Reserve University in Cleveland because he had put me up for promotion to professor at Western Reserve, which had failed to pass; he was very upset.

At the University of Minnesota, I taught a course in metabolic enzymology, the lectures of which I wrote into a short book. I worked on the two forms of GS and their characterization and was devoted to seeking or ruling out the possible role of cAMP in insulin action. Over the years, we made many friends and had a number of fine students and postdoctoral fellows at Minnesota. When offered the position of Chair of Pharmacology at the University of Virginia, Fran and I were charmed by the university, its setting, and particularly the weather, so I decided to accept. I phoned Ollie Lowry, Professor and Chair of Pharmacology at Washington University in St. Louis. Earl and I both had regularly consulted Ollie for advice. I asked Ollie what was his secret in being a chair and still continuing doing hands-on research in the laboratory. He said, "Come stay with me for a day or so in St. Louis and I will tell you," which I did. He graciously told me that his father had been in charge of the Board of Education in the city of Chicago, and that was a big administrative job. Being a chair was much easier. His father told him, "First, never say 'yes' on the telephone; second, emblaze these three words on your forehead and never forget them: ORGANIZE, DEPUTIZE and SCRUTINIZE. If you do all three, you will succeed; if you forsake either one or two, you will fail." I took this advice to heart and, at the University of Virginia, imparted it to all members of the department, several of whom went on to chairmanships.

A Long-standing Debate with Earl Sutherland: Move to Virginia

Our family moved into a newly constructed home in Charlottesville. Carlos Villar-Palasi moved with me. There were three faculty in the Department of Pharmacology: Mike Peach and Tom Westfall, both trained in the outstanding Department of Pharmacology at the University of West Virginia in sympathetic and neuroendocrine pharmacology, and Des Gourley, trained in insulin action at the University of Toronto in Canada. As already mentioned,

FIGURE 6. **Fred Murad (*left*) and James Larner (*right*), head of radiation therapy at the University of Virginia, at Joe Larner's ninetieth birthday symposium (April 2011).**

FIGURE 7. **Alfred Gilman speaks at Joe Larner's ninetieth birthday symposium (April 2011).**

my first hire was Bob Haynes from Western Reserve University, who worked on the role of cAMP in ACTH steroid synthesis. I then hired Fred Murad (Fig. 6) from NIH as head of clinical pharmacology working on cGMP action and Al Gilman from Marshall Niremberg's lab at NIH to work on G proteins involved in cAMP action (Fig. 7). I had taught both as graduate students at Western Reserve University. I then hired Ted Rall from Western Reserve, prodded by Fred and Al. Ted had been Earl's collaborator in all of the seminal work on cAMP and had mentored Fred and Al on their thesis research. The group was also very high on Gary Brooker at the University of Southern California, who had done some really nice work on cAMP phosphodiesterase. He was hired. Marty Rodbell called from NIH and said he was interested in a job but decided not to come when his wife objected: "There aren't enough sidewalks in Charlottesville." I told that story at a Rodbell memorial service in North Carolina when she was in the audience, and she remembered and had a big smile on her face.

Starting at Western Reserve University and continuing while I was at the University of Minnesota and the University of Virginia, I had a long-standing debate with Earl Sutherland about an insulin second messenger. I had a hunch that insulin also might have a second messenger analogous to cAMP, as we had found that insulin acted intracellularly to control the phosphorylation state of GS. Earl was convinced that insulin already had a second messenger; it acted by decreasing cAMP. He treated liver slices with epinephrine, first elevating cAMP, and then added insulin, which decreased the elevated cAMP, a two-hormone experiment. Carlos Villar-Palasi and I simply treated rat diaphragms with insulin alone, activating GS with no change in basal cAMP. Earl insisted we were inept analysts or that cAMP was compartmentalized.

I collaborated first with Nelson Goldberg at the University of Minnesota and then with Gary Brooker, Al Gilman, and Fred Murad at the University of Virginia, analyzing cAMP in insulin-treated tissues with the same negative results. Finally, with Laura Huang, Carlos Villar-Palasi, and others, we found that, although the basal content of cAMP was not altered by insulin, the sensitivity of the cAMP kinase to cAMP was decreased, and the cAMP kinase was maintained in an inactive holoenzyme form by insulin (32, 33). The mechanism has yet to be worked out, but, as a result, we felt that we had an assay for the putative insulin mediator, *i.e.* an inhibitor of cAMP kinase.

We injected rats with insulin under controlled conditions to minimize epinephrine release; prepared heat-inactivated muscle and liver extracts; and tested for insulin-increased cAMP kinase inhibition, which we found on the first try. Sizing columns indicated that the heat-stable material was of low molecular weight, between 1000 and 1500. Charcoal did not absorb the inhibitory material, indicating that it was not nucleotide. At this time, Len Jarett at Washington University in St. Louis was searching for an insulin second messenger released from adipocyte

cell membranes, which, when added to mitochondria, activated mitochondrial PDH. We sent him blind column fractions of our cAMP kinase inhibitor, and we published two adjoining papers in *Science* demonstrating the presence of the two bioactivities in the same column fractions (34, 35). Our lab then set up both bioassays and started to purify both bioactivities. We initially reported the presence of amino hexose as well as amino acids and mistakenly thought that they were glycopeptides (36). Other investigators claimed that they were proteolytic peptide artifacts, casting a negative spell on the field. After hearing a lecture I gave in North Carolina, Alan Saltiel and Pedro Cuatrecasas became interested and were the first to demonstrate the presence of inositol and glucosamine (37). We successfully separated and analyzed the two purified bioactive fractions from rat liver. The cAMP inhibitor contained *myo*-inositol and glucosamine (38), whereas the PDH phosphatase (PDHP) stimulator contained, to our surprise, DCI and galactosamine (39). Both the cAMP kinase inhibitor and the PDHP stimulator were insulin-mimetic *in vivo* (40). They decreased hyperglycemia dose-dependently in streptozotocin (STZ) diabetic rats and stimulated diaphragm muscle glycogen synthesis in normal controls. Thus, they were both inositol glycans and likely arose from glycosylphosphatidylinositol (GPI) lipid or proteinated precursors. We provided evidence for the latter precursor mechanism when we demonstrated that alkaline phosphatase, the first GPI-anchored protein identified, was released into the medium together with the inositol glycan activating PDHP in BC3H1 myocyte cells that had been treated with insulin (41).

Structure and Synthesis of the First *chiro*-Inositol Glycan INS-2, an Insulin Mimetic and Sensitizer

Following ten to fifteen years of effort, we finally devised a purification protocol, initially in rat liver. We scaled up and processed about 100 kg of fed (insulinized) beef liver obtained from Virginia Tech University. We obtained sufficient pure material to determine its structure by chemical analysis and two-dimensional NMR. With John Price and Tom Piccariello, we chemically synthesized the pseudo-disaccharide and named it INS-2 (42). Its novel structure is galactosamine β1,4-pinitol, where pinitol is 3-*O*-methyl-DCI. All GPI-anchored proteins studied thus far had a *myo*-inositol α1,6-linkage to glucosamine. INS-2 was therefore unique. It was isolated as a 4:1 Mn^{2+} chelate. Of interest, two-thirds of the molecule, *i.e.* Mn^{2+} and pinitol, were independently discovered as anti-diabetic agents in traditional medicine: Mn^{2+} in South Africa (43) and pinitol in India (44).

INS-2 is active as an insulin mimetic and sensitizer *in vivo* and *in vitro*. It lowers hyperglycemia dose-dependently in STZ-induced type 2 diabetic rats (42). The β-anomer is active, and the α-anomer is inactive or weakly active (42). INS-2 stimulates testosterone synthesis in ovarian theca cells as potently as insulin (45). An anti-INS-2 polyclonal antibody blocked INS-2 action as well as insulin action, indicating that INS-2 is an intermediate in insulin action (45). INS-2 stimulates glycogen synthesis in rat hepatoma cells dose-dependently (42), and it sensitizes low dose insulin to decrease hyperglycemia in STZ-induced type 1 diabetic rats (46). It has not been tested in humans.

Mechanistically, INS-2 binds in an allosteric pocket next to the catalytic site of protein phosphatases of the PPM (protein phosphatase, magnesium-dependent) family, namely PP2Cα, which dephosphorylates and activates GS (46). Activation of PDHP by INS-2 explains the mitochondrial action of insulin, as yet unexplained by the insulin receptor-stimulated phosphorylation cascade. Thus, the disposal of both non-oxidative and oxidative glucose is activated by INS-2 via phosphatase activation. Others have shown that PP2C activates PI3K by dephosphorylation of a serine on its regulatory subunit (47) and that it inactivates AMP-activated protein kinase by dephosphorylation of Thr-172, an insulin-mimetic effect (48). Thus, INS-2 is positioned to play a key regulatory role in insulin signaling by activating protein phosphatases. The x-ray crystal structure of INS-2 in the allosteric site of PP2C made the cover of the journal *Biochemistry* (46). A carbon bridge INS-2 analog was synthesized by our collaborator David Mootoo at Hunter College in New York as a possible orally available agent. It was shown to be specific for PDHP and inactive on PP2Cα (49).

Human and Animal Therapy with D-*chiro*-Inositol

chiro-Inositol itself was shown to be effective in the clinical treatment of early type 2 diabetes in the United States (50) and in South Korea (51). It is also effective in restoring ovulation in women with polycystic ovary syndrome (52). In diabetic animals, it is an antioxidant and prevents peripheral neuropathy (53, 54). Of interest, we have shown that manganese sensitizes DCI and pinitol to decrease hyperglycemia in STZ diabetic animals (55). DCI has most recently been shown to be effective in preventing neuronal damage from β-amyloid peptides in an *in vitro* model system of Alzheimer disease by enhancing insulin signaling (56). An NIH proposal for a clinical trial in early Alzheimer disease has been submitted.

Inositol Imbalance in Insulin Resistance

The rationale for DCI administration has been its deficiency in type 2 diabetic urine, type 2 diabetic muscle biopsies, and type 2 diabetic autopsy muscle (57, 58). Strikingly, this deficiency of *chiro*-inositol is accompanied by the presence of excess *myo*-inositol. We have termed this an inositol imbalance (59) and have hypothesized a defective epimerization of *myo*-inositol to *chiro*-inositol (60, 61). In addition, others have shown a lack of generation of INS-2 bioactivity in type 2 diabetic blood during a glucose tolerance test compared with controls (62), a lack of generation of INS-2 bioactivity in the blood of women with polycystic ovary syndrome compared with controls during an insulin clamp (63), and decreased INS-2 bioactivity released from placental membranes treated with insulin *in vitro* from women with preeclampsia compared with controls (64). Thus, a lack of DCI and INS-2 and an increased ratio of *myo*-inositol to *chiro*-inositol in urine and tissues are clearly associated with and are potentially markers of insulin resistance. This led to the hypothesis of decreased *myo*-inositol to *chiro*-inositol epimerase activity as a player in insulin resistance, the inversion of C3 hydroxyl (60, 61).

myo-Inositol to *chiro*-Inositol Epimerase

We investigated this hypothesis *in vivo* and then *in vitro* (60, 61). We first demonstrated *in vivo* a markedly decreased epimerization of *myo*-[^{3}H]inositol to *chiro*-[^{3}H]inositol from about 20–30% to about 5% in insulin-sensitive tissues of the Goto-Kakizaki type 2 diabetic rat inbred for insulin resistance. We then partially purified the rat liver *myo*-inositol to *chiro*-inositol epimerase enzyme and demonstrated its absolute requirement for nucleotide, indicating an oxido-reductive mechanism (61). We next demonstrated that Goto-Kakizaki type 2 diabetic tissue extracts displayed decreased *myo*-inositol to *chiro*-inositol epimerase enzyme activity compared with control Wistar rats (61). We have now prepared highly purified rat liver epimerase (19,000-fold) and submitted the preparation to LC-MS analysis to identify the epimerase gene by tryptic peptide sequence analysis. Of interest, a recent paper has stated that *chiro*-inositol is not produced in the body but comes only from the diet (65). Will scientific controversy never cease?

Ongoing Current Work

We are now involved in five ongoing projects, which we pursue even without peer-reviewed funding. 1) With Thurl Harris and Steve Rich at the University of Virginia, we seek to determine the gene(s) for the *myo*-inositol to *chiro*-inositol epimerase and understand its role in insulin resistance. 2) With Michael Thorner, Bruce Gaylinn, and Jianhua Liu at the University of Virginia, we now have a specific polyclonal antibody to INS-2, which we hope to develop into an immunoassay for insulin resistance. 3) We have isolated and partially characterized a pseudo-tetrasaccharide in the INS-2 series with ten times more potency than INS-2 *in vitro*. Its potency *in vivo* approaches that of insulin. We wish to determine its structure and to synthesize and characterize it biologically. We will collaborate with David Brautigan at the University of Virginia and David Mootoo at Hunter College in this project. 4) With Chien Li at the University of Virginia, we have shown that that INS-2 stimulates insulin secretion from the murine pancreatic beta cell line MIN6 by directly acting on the ATP-inhibited K^+ channel by a novel mechanism. Sulfonylureas bind and act on the sulfonylurea receptor binding subunit. 5) With Susan Aja and Gabriele Ronnett at The Johns Hopkins University School of Medicine, we have shown that INS-2 administered intracerebrally in mice decreases appetite, food intake, and body weight by acting locally in the brain. Further mechanistic studies are under way.

Conclusion

All in all, I think that Earl Sutherland would have been pleased to live to see the positive outcome of our argument, especially because we both successfully isolated allosteric small-molecule regulators of the phosphorylation-signaling cascades. Earl's acts on kinases and ours on phosphatases involved in glucose disposal via oxidation and glycogen synthesis. Also, I think that Herb Carter would have been pleased to learn of the many important roles of inositols, including inositol trisphosphate, inositol bisphosphate, inositol pentakisphosphate, and INS-2, in insulin signaling and metabolic control.

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