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RECOLLECTIONS

Linderstrøm-Lang and the Carlsberg Laboratory: The view of a postdoctoral fellow in 1954

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Although we carefully avoid any mention of it in publications, and do our best to keep it out of the public image of science, chance and fad play a strong role in both the research and the career of any scientist. In my own case these factors carried me into an unusual graduate program and the opportunity to work under two very different mentors during my postdoctoral period.

Following completion of an undergraduate degree in chemistry from MIT in 1948 and some conversations with my oldest sister, already a biochemist, I joined the Department of Physical Chemistry at the Harvard Medical School, the only such department in any medical school then or since as far as I know. This casual decision was made without any survey of other opportunities. I spoke to John Edsall and Larry Oncley and they said to come along if I wanted to. At that time there were no training grants and no evidence of competition for student "slots." There was no "athlete style" recruiting either, a phenomenon in graduate science education that began somewhat later. The golden age in what would be called molecular biology was just beginning.

This department had been started in the 1920s by Edwin Cohn and was disbanded in the late 1950s after his death. Cohn was a brilliant man, a first-class scientist, an excellent organizer, and a major-league tyrant. He talked with his faculty, his staff, and the postdoctoral fellows. Although he was polite enough, it was never quite clear where graduate students fitted, if at all, into his scheme of things. There were very few of us, and for registration and course records we were lumped in with the students in the Biochemistry Department. Thus, we did not present any administrative problems for Cohn. John Edsall and Larry Oncley took on the major responsibilities for our education.

A huge group had been assembled during the Second World War by Cohn to develop blood fractionation procedures, which were enormously successful and saved countless lives with their use in military medicine. There was a very close connection between the Cohn laboratory and the commercial sector, where the blood fractions were produced in quantity. One of these industrial concerns was the Armour company, which most of us associated then and now only with hot dogs. More on this later. In the early 1950s Cohn's laboratory was shrinking from its peak wartime population but was still quite large and prominent.

For the proper perspective on the rest of this discussion it is important to remember the status of the protein field in the early 1950s. The sorting out of the previous three decades was still underway. In the 1920s and 1930s there were major arguments about whether proteins were or were not macromolecules. This was the colloid era, and many thought that they were just aggregates of smaller compounds. It was not clear that the peptide bond was the central feature of native proteins even though in the denatured state one certainly could derive peptides from them. Until Fred Sanger's work in the late 1940s it was not known whether the peptide chain, if it existed, had a unique sequence or not. The best one could do in describing size and shape was to consider a protein as a sphere, ellipsoid, or rod. No detailed structure was available for any protein. The Pauling and Corey papers on models for the alpha-helix and beta-sheet appeared in 1951. Watson and Crick proposed a structure for DNA in 1953. The central dogma on the relations between

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DNA, RNA, and protein was only starting to be developed. The first single crystal X-ray structure, myoglobin, would not appear until 1959. Today we have other problems to solve, and it must be hard for current students to imagine the uncertainty and confusion that existed in the early 1950s on many of these fundamental issues.

My own thesis advisor was Barbara Low, then a relatively new assistant professor fresh from her very successful work with Dorothy Crowfoot Hodgkin in England on the structure of penicillin. Cohn had the wisdom to realize that the details of protein structure were most likely to come eventually from X-ray crystallography. By chance I had taken a full-year physics course in X-ray diffraction and crystallography with Bert Warren at MIT as an undergraduate elective. Playing with protein crystals, which were then being grown in Cohn's department, seemed like a fun thing to do for a thesis. The best one could hope for was to determine an accurate molecular weight. Sequence data for proteins other than insulin were not available. There were many uncertainties about the interpretation of the results from the various physicochemical procedures leading to estimates of size and shape, so an unequivocal measurement of molecular weight was not an unreasonable goal at that time.

After determining the space group of a crystal, measurement of the unit cell dimensions was straightforward. The mass density and composition of the crystals became the major roadblocks to the molecular weight estimate. Micromethods were needed. Chris Anfinsen was at Harvard at that time and suggested that we try the density gradient column, which had been invented in Denmark by Linderstrøm-Lang some years earlier for a different purpose. It turned out to work very well indeed.

With some trepidation I decided to spend a postdoctoral year in 1953 as an employee of the Department under Cohn's personal supervision. As a postdoctoral employee, I now saw and spoke to him regularly and eavesdropped on some of his meetings with others. Although the conversations tended to be a bit one-sided, I did get to see why he was such a towering figure in the field of biochemistry in that era. It was a very interesting and useful year, providing two quite different kinds of experience: immersion in protein chemistry and learning to deal with a domineering personality.

Toward the end of my graduate work, one of the many visitors that were always appearing to see Cohn or Edsall or Oncley was Kaj Ulrik Linderstrøm-Lang, referred to by all just as Lang. Needless to say, I asked to have a chat with him since he was indirectly responsible for a major part of my thesis. What a delightful individual, full of fun and jokes as well as science! Although both Lang and Cohn were strong and forceful men, it is hard to imagine two more different personalities. Because by then Lang's laboratory was world famous for its contributions to the study of proteins and also for the development of micromethods, it seemed the obvious place to go for a postdoctoral stint. In response to my query he said that there might not be any space and certainly no money for a stipend, but by all means come if I could. The decision to go was based on this brief contact, some equally brief conversations with Barbara, Chris, and John, and some help from the National Research Council. It did not involve a careful investigation of the country, the laboratory, or the man. It was an accident. The background details I only found out much later.

The Carlsberg Laboratory was and is a remarkable institution (Fig. 1). The Carlsberg Brewery was started by J.C. Jacobsen. By the 1870s this man was not only very successful in business but was impressed through the work of Louis Pasteur by the possible impacts of science on human civilization. With great foresight he set up the Carlsberg Foundation, which was fed from the profits of the Brewery. The Foundation in turn initiated a series of philanthropic activities in Denmark, a major component of which was the Carlsberg Laboratory, started in 1876. This was to be a basic research laboratory with its own agenda

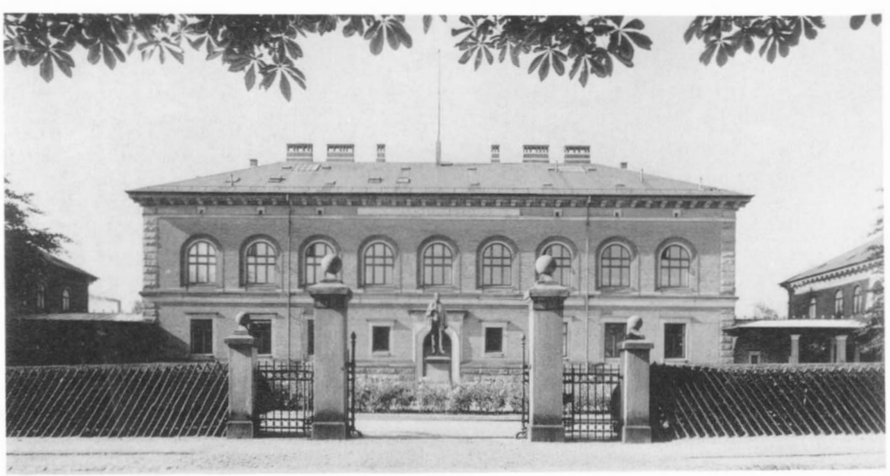

Fig. 1. Number 10 Gamle Carlsbergvej, The Carlsberg Laboratory, main building (picture taken in 1976). The Director's house is attached at the left. (Courtesy of Carlsberg Foundation Picture Archives, Copenhagen.)

and not the process control laboratory, which was a separate unit at Carlsberg. From the start the Laboratory has had two Departments, Physiology and Chemistry, each with its own record of very unusual people. The whole history of this organization is recorded in the fascinating Centenary Volume edited by Heinz Holter and Knud Max Møller (1976). (Just think how the support of science would be altered if a significant fraction of the world's breweries were to take such an enlightened approach to the distribution of their profits!)

The Chemistry Department has had a series of very distinguished Directors who have all left their mark on protein chemistry. The first was Johan Kjeldahl, who developed the procedure for the measurement of total nitrogen content that bears his name. This procedure was important in chemistry in general, but particularly in the protein field, where accurate analytical procedures were few and far between at that time. It was the major analytical technique for organic nitrogen for decades. Kjeldahl was succeeded in 1901 by S.P.L. Sørensen, who held this position for the next 37 years. His work centered on the chemical synthesis of amino acids, the development of precise analytical procedures, the measurement of hydrogen ion concentration and titrations, and studies on the isolation and purification of proteins and enzymes. He invented the logarithmic pH scale and the early colorimetric methods for pH measurement. He demonstrated the enormous importance of pH in biological function, especially enzyme activity. His work turned biochemistry into a quantitative science. Sørensen was a kindly man, but he demanded meticulous care in experimentation and carefully recorded the problems as well as the successes with his techniques. He focused on experiments and was reluctant to enter into theory.

Kaj Ulrik Linderstrøm-Lang began his career as Sørensen's assistant in 1919. He absorbed the culture of the laboratory—meticulous attention to experimental detail and proper treatment of one's colleagues—but brought much of his own as well. He was always interested in theory and had a strong mathematical bent. He was an artist, a musician, a playwright, an author, and a storyteller. As with so many great scientists, at an early stage in his career he might easily have become a professional musician or artist rather than a scientist. The decision appears to have been the result of an accumulation of factors, but among them the need for a paying job was a major concern. As it turned out Lang spent his entire scientific career at the Carlsberg Laboratory. He succeeded Sørensen as Director in 1938, a position that he held until his unfortunate death at the age of 62 in the spring of 1959.

By 1959 there was a long list of enthusiastic "alumni/ae" of the Carlsberg Laboratory. Lang had been in the top rank in international stature for years and was both admired and loved. His death came as a great shock to all. Obituary notices appeared in many places and catalogued in detail his life, his science, and his honors. The articles that happen to be in my reprint collection are by his great friend and colleague Heinz Holter (1960) (this paper is also reprinted in the Centenary Volume); by Martin Ottesen (1959), Lang's first assistant and successor Director of the Chemistry Department; and by the Americans Hans Neurath (1960) and John Edsall (1959). I recommend any and all of these to the interested reader and acknowledge my indebtedness to these authors for much of the information in this paper. No attempt is made in this short report to provide a comprehensive account of Lang's work. Some overview comments and some items of particular relevance to me in 1954 are noted below.

In 1924, at the age of 28, Lang published a seminal paper "On the Ionization of Proteins." This appeared only 1 year after the announcement of the Debye–Huckel theory of solutions of electrolytes. Extending this new view of ionic solutions, Lang derived the equations for the titration curve of a protein. With the knowledge available at that time he had to assume a spherical molecule, and not knowing where the charges were he smeared them on the surface. He recognized that any ionizable macromolecule, even at fixed pH, is not a unique species but an ensemble of fluctuating charged states. He quite accurately predicted the influence of ionic strength on the titration curve and made the distinction between the isoelectric and isoionic points. Except for refinements by John G. Kirkwood and Charles Tanford in the 1950s, Lang's approach was not improved upon until the actual atomic-level structures and localization of specific charges became available with the success of the X-ray studies that began to appear just after his death.

He made use of changes in the number of ionizing groups and the resulting electrostriction effects in various enzyme assays, for example, the appearance of new amino and carboxyl groups on hydrolysis of a peptide bond. The decrease in volume produced by electrostriction around the new ions resulted in a slight change in the density of the solution. He was able to measure this contraction either by dilatometry for large samples or by the gradient column procedure, which he invented for tiny samples. In a properly functioning constant temperature bath a gradient between two organic liquids of differing density can be stable for months because macroscopic diffusion is a very slow process. With a density of 0.99 g/mL for the top liquid and 1.01 for the bottom liquid, the density difference of 0.02 g/mL was spread out over 20 cm in the column. This gave a gradient of 1×10^{-4} g/mL/mm. The position of a small drop in the column could be estimated easily to 0.01 mm. Thus, the sensitivity of this instrument was about 1×10^{-6} g/mL. This remarkable precision was obtained for the price of a water bath and a piece of glass tubing (Fig. 2).

The gradient tube was designed to measure enzyme kinetics in small pieces of tissue in microliter size drops. In the early 1950s Lang was using the same equipment to measure the deuterium content of water samples in his

Fig. 2. Linderstrøm-Lang loading a water sample on a gradient column with a Carlsberg constriction pipette during a hydrogen exchange experiment in 1951. The deuterium content was calculated from the density of the liquid, which could be estimated to 1 part in 10^6 in this apparatus. (Courtesy of Carlsberg Foundation Picture Archives, Copenhagen.)

ingenious development of amide hydrogen-exchange kinetics for the determination of secondary and tertiary structure in proteins. He developed both the in- and out-exchange techniques. The exchange reactions were stopped by freezing the solutions at −60 °C. The water was "cryosublimed" from the sample, melted, and its density measured in a gradient column, yielding the isotopic content. The composition of a water sample with about 2 parts of D_2O in 1,000 parts of H_2O could be measured with an accuracy of 1%. Knowing the total amount of deuterium in the sample, the isotopic content of the protein could be calculated by difference. No mass spectrometer, no NMR machine. (In fact, in 1954 nuclear magnetic resonance, only recently discovered as a property of certain atoms, was just on the horizon for any applications in chemistry.)

In a long series of studies Lang and Aase Hvidt first confirmed the fast exchange of N- and O-bound protons in smallish peptides. The slowing of the exchange in alpha-helices, including the effects of end fraying, was then established, and finally the dramatic slowing produced by the formation of tertiary structure from which they inferred stabilization of the secondary structure. The quantitative estimates of the number of protons in various exchange rate classes that were obtained from the data could really be evaluated only for insulin, the single protein whose sequence was available at that time. Of course, specific proton identification, now possible in certain systems, was unapproachable in the 1950s.

After the success of the crystallographic procedures, the amide exchange procedure was eclipsed for about two decades. It was kept alive and further developed almost single-handedly by Walter Englander and his laboratory during that period. The availability of tritium added a whole new dimension to exchange studies. Today, particularly through NMR, there is a resurgence in the use of exchange measurements, particularly as an effective general probe of dynamic behavior. Such studies are especially useful in cases where the X-ray structure is also known. The techniques are complementary because the fluctuations whose dynamics are probed by hydrogen exchange are largely inaccessible through crystallography.

After World War II one of Lang's major efforts was the study of the action of proteolytic enzymes, not in the biochemical sense of peptide bond specificity, but on the general mechanisms by which native proteins were attacked, on the various possibilities for intermediate states, and on what one should expect to see in actual experiments. The studies involved another clever experimental procedure developed at Carlsberg, the pH-stat. Almost all biochemical reactions cause the uptake or release of protons either directly as in hydrolysis reactions or indirectly through changes in the ionization behavior of neighboring groups. The pH-stat is a mechanical buffer (Fig. 3). The pH meter is connected through appropriate relays to a motor-driven syringe, which adds acid or base to the reaction solution to maintain the pH at the selected value. The first models simply had fine wires making electrical contact with the meter needle. The recorder plot of the amount of acid or base added as a function of time is an accurate record of the kinetics of the reaction. The pH can be maintained to about ±0.001 units without undue effort. Thus, a very small proton signal can be monitored. Note that such information is normally lost when chemical buffers are used and their pH control is nowhere near as good. This procedure is admirably suited to the study of proteolytic digestion.

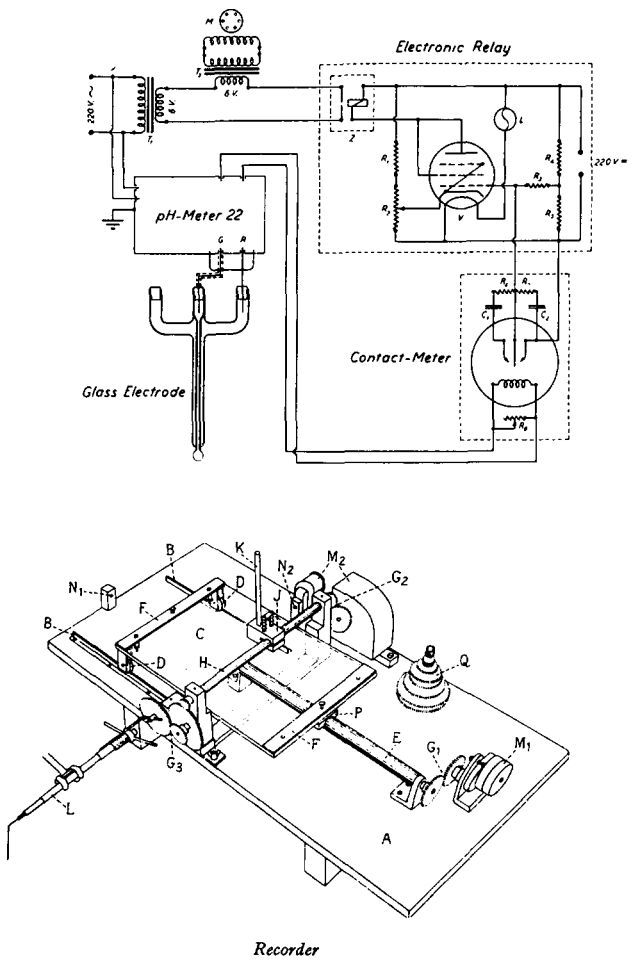

Fig. 3. Schematic diagram of the original pH-stat (Jacobsen & Leonis, 1951). The contact meter, in parallel with the pH meter, sends a signal to the motor, which drives the syringe and the pencil on the homemade XY plotter. The latter records as a function of time the volume of acid or base added to maintain the pH. (Reprinted from Jacobsen and Leonis [1951].)

His work in this area was presented in the Lane Medical Lectures at Stanford in 1952 (Linderstrøm-Lang, 1952). It was here that he presented and defined in detail the concepts (and names) of the primary, secondary, and tertiary structure of a peptide chain. The concepts and names survive to this day. Much of his experimental work on enzymes and earlier studies with Heinz Holter at the cell biological level, where many of the micromethods, such as the gradient column and the Cartesian diver, were developed, are also covered in these lectures.

Unaware of the above information, my wife Heidi and I and two small daughters arrived at Kastrup airport on December 13, 1953. Our crash course in Danish the previous month produced just enough glottal stops to get us to the pension on Vestersøgade, which took care of us for the next 2 weeks. The following day I made my first visit to the Carlsberg Laboratory at 10 Gamle Carlsbergvej. The marvelous Lise Allen who, as far as I know, took care of all the nonscientific business for everyone, especially foreign visitors, dug Lang out from his experiments. We had a couple of hours discussion on what was going on in the laboratory and what might make a good project for the year. He also said to be sure to come to the lab Christmas party on December 21.

What a party! My somewhat vague memory was a cast of thousands, although I expect there were on the order of 100 people: all the lab personnel with spouses, members of other labs in Copenhagen, all sorts of visitors, and particularly Fritz Lipmann, who was returning to the States from Stockholm, having just received the Nobel Prize a few days earlier. All of these were gathered in the great entrance hall that had been set with tables for the evening. The "vagueness" was produced by the food, beer, schnapps, and smoke, all of which appeared immediately upon entering since Danish protocol requires that everyone arrive precisely on time. The singing was frequent and not bad, at least initially. By the time the coffee was cleared away and the serious drinking began, Lang was in his shirtsleeves, flip chart on its easel, and crayon in hand. Caricatures were flying off the easel at least one a minute.[1] Two of them are shown in Figure 4, one of Lipmann and the other of the author. In compar-

[1] This description of the memory of the author is accurate. However, the memory is, in fact, incorrect and serves as an example of the frequent unreliability of eyewitnesses. Knud Max Møller, who, as assistant to Lang, had attended many of these parties, assures me that the sketches were actually made in his office in a more leisurely fashion the day before the party, and then hung in the great hall for the following evening.

Fig. 4. Sketches by Linderstrøm-Lang made for the Christmas party on December 21, 1953. Left panel, Fritz Lipmann; right panel, Fred Richards. See text for further description. (Courtesy of Carlsberg Foundation Picture Archives, Copenhagen.)

ison with an actual photograph, one might say that the chin of the latter was somewhat exaggerated. However, as many Yale Administrations over the last 35 years would confirm, this was just another example of Lang's genius, the ability to combine both the physical and behavioral aspects of the individual in a single instant sketch. As an introduction to science and schnapps in Denmark, this party was a real winner. The party also turned out to be an excellent example of Lang in action.

He loved to take guests to Tivoli, that marvelous institution in the center of Copenhagen where during an evening you can ride on a roller coaster, shoot moving targets for prizes, buy all sorts of unusual items, eat at any one of a number of first-class restaurants (always with ice-cold aquavit and beer of course), listen to a symphony orchestra, and close out the evening at midnight with a spectacular show of fireworks. Following Lang, many lab members became addicted to Tivoli.

In 1957 at the New Hampton School in New Hampshire, Lang's sense of mischief caused a "serious" problem at the Proteins Gordon Conference. Closing up the bar in the wee hours, he led those stalwarts still standing to the school bell and proceeded to ring it loudly for about a minute. The next morning Mr. Madan (whom many of the older members of our field will remember well) an ex sergeant major with an enormous voice who must have terrified the boys during the school year, assembled the entire Conference, dressed them down, and demanded to know who was responsible for the previous evening's activities. Lang, of course, confessed and to our great surprise managed to mollify Madan. Much amusement all around. This streak in Lang's character shows up in his writing as well. Two of his well-known contributions, "Taxi Drivers in New York" and "The Thermodynamic Activity of the Male Housefly" are reprinted in his *Selected Papers* (1962). Do read them.

After finding a house to rent for the year, the regular work at the lab began with a detailed look to see what was there and where everything was. The desk I was assigned was just outside the door to the Director's house. Nosing into the cabinets under the lab bench, what should appear but some of the original Kjeldahl flasks and the burner rack used for heating the digests. These items were then about 70 years old and still perfectly usable. In another area was the multidrawer cabinet for the standard volumetric pipettes, which were common stock for everyone. Casually opening the drawer, I noticed that some of the tips were broken. More careful examination showed that all the tips were broken. Glassware was too expensive to throw out. In many cases one did not need the full potential accuracy of the pipette. If you did, you were expected to firepolish the tip and recalibrate it. If, for some reason, a pipette was required with the specific labeled volume, you went to the first assistant, who unlocked a special cabinet and carefully handed you an unbroken pipette, which you were expected to return in the same form as soon as you were through with it. The fanciest commercial equipment that I found on the first tour was a Beckman DU spectrophotometer.

What a change from the Cohn laboratory! During the Second World War Cohn had a very high priority for scientific equipment because of the military importance

of the blood fractionation work. Each investigator had essentially all the equipment one might want. Standard laboratory glassware and all the small equipment items were always available. Broken glassware was simply discarded. Excellent machine shop facilities with a full-time instrument maker were mandatory because much of the equipment was complex and home made, for example, an analytical ultracentrifuge and a Tiselius free boundary electrophoresis apparatus. (The Spinco company and its commercial versions of these machines did not appear until later. Even these commercial versions have now disappeared almost completely. There are only a few of today's graduate students who have ever seen them.)

During the second war, the Germans occupied Denmark. The Carlsberg Laboratory was not damaged, but it was impossible to get any equipment or supplies. By the end of the war, the laboratory was short of almost everything, and the normal suppliers were only slowly reactivating their businesses all over Europe. Several of the Carlsberg alumni in the States got together and with their personal funds bought a small amount of volumetric glassware that was sent to Copenhagen to help the rebuilding process. The shipment was carefully preserved. Lang had remained at the lab during the war, but he was a very active member of the resistance movement in Denmark. Although arrested once, by skill and good luck he otherwise avoided detection and its corollary, almost certain execution.

Lunch was an important component of the daily routine at the lab. Everyone ate together in the basement dining room. It was always a jolly affair with much talk and occasional competition with "Carlsberg Rockets" (the projectiles produced from wooden matches and the tin foil from inside the bottle caps). You brought your own lunch, but the beer or soft drinks were free. These were not only available during lunch but during the whole day and were free as long as they were drunk on the premises. Every few days a horse-drawn wagon from the Brewery across the street would draw up in front of the Laboratory, and the driver and his helper would replenish the stock in the coolers. One day Lang banged his glass and stood up to make a speech. This was very unusual and Lang was obviously very solemn, quite unlike his usual self. He announced in sepulchral tones that he had received a communication from the Brewery. The Administration was concerned about the beer consumption at the lab, and with regret was going to have to limit the deliveries. From now on each individual was going to be restricted to six bottles per day. Dead silence. Rapid mental calculations. Gradual sighs of relief. (Note that the personnel were roughly equally divided by sex and that many of the ladies drank only the soft drinks, which were not part of the restriction.) Eventually only Ieuan Harris, a Welsh visitor from the MRC Laboratory in Cambridge, was left with a frown. He had been upholding the Welsh tradition with beer and was not certain he could get by. Since the shelf above his bench was filled with a double row of empty bottles for a distance of at least 8 feet, we understood his problem. Offers from various of the men to share their daily ration finally relieved his anxiety and normal conversation resumed. Because of his elfin smile, the exact extent of Lang's personal concern was never quite clear, but he did love his beer.

The Director's house is attached to the Laboratory. Every morning Lang would enter the lab from the connecting corridor and begin his tour. He invariably had a cigar in his hand, lit, and with about an inch of hot ash. The first six or eight steps would bring him to the sink in the first lab. Here, without looking, the ash was neatly deposited in the drain. Ieuan Harris was carrying out the Sanger FDNB endgroup procedure on an adjacent bench. He would extract the dinitrophenyl amino acid derivative with ether, and this solvent eventually was discarded in the sink. Not infrequently the hot ash found a residue of this solvent. The resultant whoooooosh and brief flame was always a shock for new visitors. Lang took no notice and would continue his tour, talking with the individuals that he happened to find. Of course, had it existed in Denmark in those days, OSHA would have shut down the lab.

C.F. Jacobsen, Lang's first assistant in the late 1940s, had made elegant detailed studies of the proteolysis of chymotrypsinogen to yield the various forms of chymotrypsin, thus starting a long-standing interest of the laboratory in limited proteolysis (Linderstrøm-Lang, 1953). One day he noticed in the cold room a bottle supposed to contain ovalbumin crystals in their mother liquor, but the protective toluene layer had evaporated. Under the microscope these crystals looked unusual: instead of chunky bits, the crystals were in the shape of thin plates. Lang agreed that this was odd and asked Martin Ottesen to follow it up and see what he could find out. This led to the discovery of the new proteolytic enzyme subtilisin, so named when its origin was found to be a strain of *Bacillus subtilis* that was contaminating the ovalbumin sample. Ottesen then went on to isolate and purify subtilisin and to develop the intriguing story of limited proteolysis in the ovalbumin system. This new proteolytic enzyme and its close relatives have gone on to serve well many laboratories and industrial companies, but few better than that of the author.

In early 1954 Chris Anfinsen was in the middle of one of his many visits to the Carlsberg Laboratory. This time he had appeared with a bottle containing 10 g of crystalline bovine pancreatic ribonuclease! This very large sample was given to him free by the Armour company. After the war and with the expertise in protein chemistry developed during the collaboration with Cohn on the blood fractionation work, Armour used the procedures, then recently developed by Moses Kunitz at the Rockefeller Institute, to prepare 1 kg of the crystalline enzyme from their normal slaughterhouse material. Samples of this single lot were given out free to any laboratory in the world

that could demonstrate a need and a reasonable research project. It is hard to overemphasize the significance of this decision. Over the years a large number of groups were able to move forward the frontiers of research in enzyme structure and mechanism and to compare their results knowing that the starting material in each project was identical. There were no strings attached to the gifts. The company only asked that good science be done. For most academic biochemists, contracts and patenting were not even on the horizon in those days. Nirvana had we only known it!

Chris kindly doled out aliquots of his precious hoard to other members of the laboratory. Sumner Kalman (and Richards), Bill Harrington, and John Schellman each got some and went off on their various experiments. When Chris ended this particular stay and returned to the US, he still had most of the ribonuclease in his bottle. None of us used very much. As is well known, Chris made good use of the rest during the next decade (see the Nobel Lecture [Anfinsen, 1973]).

Bill, John, and Chris did some joint work on the denaturation of ribonuclease and its effect on the structure and activity of the enzyme on RNA. The experiments were very carefully done and were quite correct (Fig. 5A). However, the conclusion, that the denatured form of the enzyme was active, was later shown to be wrong. The substrate actually caused the refolding of the enzyme into its native, active state, a phenomenon commonly recognized today, but the necessary techniques to determine the structure of the enzyme in the presence of the substrate and denaturant were not available at that time. (Today, if this had been a U.S. Government-supported project, the latter fact might be enough to turn off an accusation of misuse of public funds, but one would be nervous [see the description by Anfinsen, 1989].)

In one of his other studies John Schellman was considered to be a clear masochist. He was in the process of developing the use of optical rotatory dispersion (later to be upgraded to circular dichroism) as a technique for estimating secondary structure. This required sitting for many hours in a specially constructed dark cubicle and staring at, and trying to match, the dim fields in a manually operated optical polarimeter illuminated by a sodium vapor lamp. The rest of that story is well-recorded history, as are the many other studies that he and Charlotte (Green) carried out during their long 3-year stay at the lab. This stay was punctuated by their marriage in February 1954 following the famous Christmas party.

In our talk about possible research projects Lang discussed his interest in the mechanism of proteolysis. In his extensive derivations of the properties of different possible mechanisms, it had become clear that a critical experiment for defining the mechanism would be measurement of the concentration of the native protein as a function of the extent of proteolysis. How might this be done? Initially it seemed that the activity of an enzyme might be a good

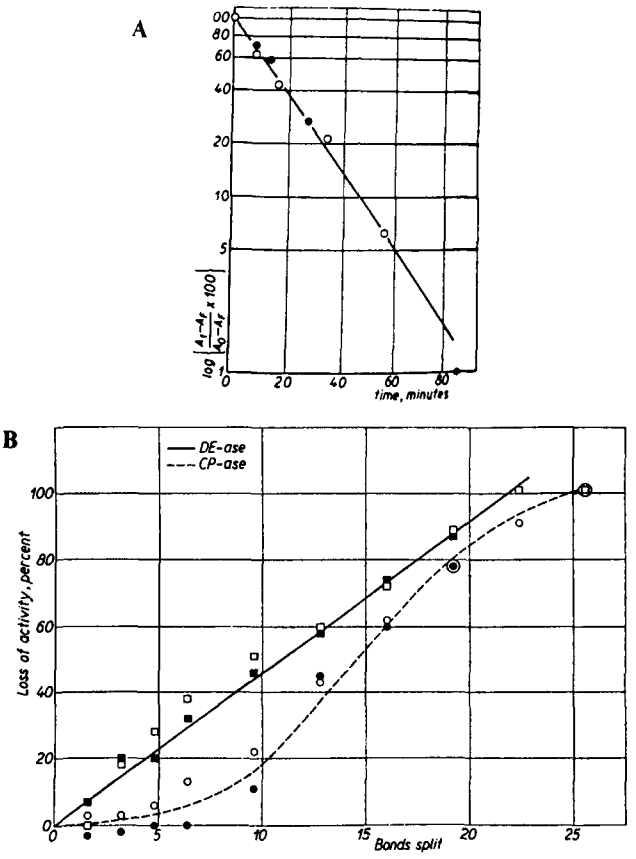

Fig. 5. Figures from two papers on pancreatic ribonuclease describing work carried out during 1954. **A**: First-order kinetic plot of the change in viscosity on digestion of RNA with (open circles) or without (solid circles) 8 M urea. Conclusion: Denatured RNase is as active as the native form (Anfinsen et al., 1955). **B**: The relative RNA cleaving activities of RNase in step 1 (diesterase, DE) and step 2 (cyclic phosphatase, CP) during digestion of RNase by the proteolytic enzyme subtilisin. Conclusion: There is a partially digested active intermediate of RNase (Kalman et al., 1955). Facts: For A the experiments are correct and reproducible, but the conclusion is wrong; for B the experiments are probably suspect since no explanation has ever been found for the apparent activity differences because the native enzyme and the isolated intermediate have the same activity in both assays, but the principal conclusion is correct. Although each paper is flawed, both led quickly to some very interesting studies by many laboratories in subsequent years. (Reprinted from Anfinsen et al. [1955] and from Kalman et al. [1955].)

measure. It seemed reasonable at that time to assume that a single bond cleavage in a molecule of an enzyme would cause its complete inactivation. Thus, an activity measurement on a solution could be converted directly to the concentration of the native, unproteolyzed enzyme. Sumner Kalman, who was visiting the lab in 1953, made a start on this project using subtilisin as the proteolytic enzyme and ribonuclease as the "substrate" enzyme. He ran into problems with RNA as the substrate for the measurement of ribonuclease activity and also with a deadline for departure due to commitments to his position in the States. Because I had arrived just as Sumner was leaving, Lang

suggested that I pick up the problem and carry on with a new twist.

In the late fall of 1953 Lang had returned from a trip to Cambridge with a precious sample of 20 mg of uridine-2′,3′-cyclic phosphate, which he had been given by Alexander Todd, in whose group it had recently been synthesized. It was known that this compound was an intermediate in the cleavage of RNA by pancreatic ribonuclease, and that it was further hydrolyzed by the enzyme in a second step to yield 3′-UMP. He suggested that I develop an appropriate assay for the enzyme with this substrate, which would thus test the second step in nucleolysis to complement the RNA assay, which depended on the first step only.

This was a fun challenge, as I expect Lang knew it would be. One had to develop the assay and then use it and in the process make sure that the whole project did not use more than 20 mg of the uridine cyclic phosphate. This was done. From broken quartz cuvettes we constructed an optical reaction cell that required 10 μL of solution. Although the reaction conditions were not extensively surveyed, we found solvent conditions that were satisfactory for the assay. Many subtilisin digests of RNase were carried out and the RNase activity followed. At the end of the year I was pleased to be able to hand back to Lang for use in other studies 10 mg of the original cUMP sample. Although I had not attained the extreme level of microprocedure developed by Lang in earlier years, it was satisfying to me and I think to him as well.

The results of this work were published, and the figure in the first short paper shows two curves for the two activities as a function of extent of proteolysis (Fig. 5B). These curves were not coincident. The immediate conclusions were that (1) the two activities were differentially affected by proteolysis; (2) because of this, there must be an intermediate form of the enzyme that was active in one procedure and inactive, or at least less active, in the other; and, (3) because of this complexity, the use of enzymatic activity to estimate the amount of native, uncleaved enzyme was not possible by this procedure, at least in this system. This conclusion was a setback for Lang's original goal, but it shifted the work to a whole new project, the attempt to isolate and characterize the active ribonuclease intermediate.

This second stage of the project used up the rest of my year at Carlsberg. Employing the column procedures initiated by Stein and Moore, the intermediate was isolated. Its activity against both RNA and cUMP appeared to be in every way identical to that of the native enzyme. If this is true, how did one explain the separation of the activity measurements on the two separate steps reported in the first paper? These data have never been explained. Was it an example of fabrication? The conclusion that there was an intermediate in the subtilisin digestion of RNase was correct. (This example is the mirror image of the denaturation work of Schellman, Harrington, and Anfinsen noted above.) No attempt has ever been made to duplicate the data on which the conclusion was made since interest turned to other properties of the intermediate now known as ribonuclease-S. If the statute of limitations had not run out, perhaps the Office of Scientific Integrity might have instituted an investigation because the Carlsberg Laboratory was receiving a small amount of NIH support at that time.

Using the pH-stat it was possible to show that subtilisin cleaved only a single peptide bond in converting RNase-A to RNase-S. Both on the ion exchange columns and on electrophoresis, the early stages of proteolysis showed only two components, the native RNase-A and RNase-S in changing ratios. There was no third component. After oxidation with performic acid to cleave the disulfide bonds, RNase-A showed a single band on electrophoresis whereas RNase-S showed two. All enzymatic activity, of course, was lost in this procedure. The conclusion for both Lang and myself was obvious: the two parts of RNase-S were held together by a disulfide bond.

At this point the postdoctoral year was over. Joe Fruton offered me a faculty position at Yale, which I accepted by return mail. That was easy because there were no negotiations for setup funds or lab space in those days. The extreme promptness was caused by the fact that I was responding to his *second* letter, asking why I had not responded to his first one, which, unbeknownst to him, had never arrived. Lang kindly let me continue with the ribonuclease problem in my new position. We both thought that it was just a cleanup operation: do the amino acid analysis of the two pieces of RNase-S with the new analytical procedures recently developed by Stein and Moore. This would permit one to identify the peptide bond that was cleaved, because shortly both Stein and Moore and Anfinsen would have the sequence of the enzyme worked out. With a little luck one would also know which disulfide bond was holding the two parts together.

Of course, it did not turn out that way. The smaller of the two pieces of RNase-S, the 20-residue S-peptide, did not contain any cystine. The two pieces could be separated by procedures that did not involve the irreversible changes of performic acid oxidation, thus permitting activity measurements on both the separate pieces and their complex. The rest of this story involves the attainment of tenure at Yale and is irrelevant to this article except for the nagging thought of what would have happened if Lang had *not* allowed me to continue with the problem outside of Carlsberg!

By 1960 it was clear to me that (1) chance and fads play a controlling role in science, and (2) there are many ways to carry on research and to run a laboratory. Each individual develops his or her own style. For the success of science one is not necessarily better or worse than the other, just different. However, the enjoyment of the members of the laboratory can be strongly affected by the per-

vading style. Today's emphasis on expensive equipment, thus money and its attendant Congressional oversight, and the constant clamor from the press over misconduct certainly pollute the research environment. Some of us yearn not for the past but for simple, inexpensive, ingenious, and insightful experiments as exemplified in the life of Kaj Linderstrøm-Lang.

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