Protein Science (1994), *3*:857–861. Cambridge University Press. Printed in the USA.

RECOLLECTIONS

Macromolecules

CHARLES TANFORD
Duke University Medical Center, Durham, North Carolina 27710

Protein chemistry had barely emerged from infancy when, almost 50 years ago, I began to be a participant. Proteins were accepted by almost everyone to be true macromolecules, hundreds or thousands of amino acids covalently linked into polypeptide chains. There were known to be at least two categories: "fibrous" and "globular." The latter category included most water-soluble proteins and most enzymes. They were so named because they appeared by several criteria to be folded into tight little globules, almost spheres, far smaller in diameter than the length of their constituent polypeptide chain or chains. Several of them had been crystallized; the crystals gave sharp diffraction patterns indicative of a high degree of order. But little was known beyond that. No amino acid sequences were known—indeed, constancy of composition or amino acid sequence for a given protein was still seriously questioned, under the heading "microheterogeneity."

Most important of all, the role of DNA as the progenitor of protein structure was not yet even guessed at. Many proteins were commonly available or easily purified in the laboratory, but we didn't have the least idea how they were made in the cell or how the information for making them was passed from generation to generation. It was widely believed that all genetic information was in fact encoded within proteins, a belief that incorporated some vaguely defined template mechanism by which a new protein molecule was "copied" from an existing one.

So how did we get to our present state of mature knowledge in a period paralleling my own scientific life? More particularly—and the title of this series of articles, "recollections," presumably invites me to ask the question—what did I myself contribute to this spectacular maturation process? To be realistic, in conventional terms of successive advances, one building upon the other, I didn't contribute much. Primary, secondary, and tertiary structures; establishment of DNA as the genetic material; transcription and translation and regulation of biosynthesis; mechanism of function—they appeared on the scene one after another without my help. I was part of a side show, peripheral to the main action. I wrote a book or two that people found useful—but that accomplishment hardly fits the popular image of a scientist, ever striving to push back the frontiers of knowledge.

I was a physical chemist by training, theoretically oriented, imbued with the knowledge that chemical molecules must rigorously obey the laws of physics. Taking one's cue from the history of physical chemistry in general (gases, solutions of small molecules), it was easy to believe that almost any of a huge variety of physical properties of macromolecular solutions should be able to reveal new insight into macromolecular structure and behavior and (in the long run) biological function. I was not thinking at all about direct determination of structure—I wasn't looking for a "picture" for its own sake. I aspired instead to what I had been taught was the more elegant process of using equations: equations that linked thermodynamic properties, transport measurements, dielectric constant and dipole moment, binding equilibria, and a host of other possibilities to revealing molecular characteristics. I wanted a "picture" of sorts, but one that emerged deductively, not by direct visualization.

The laws governing such physical quantities and their relation to macromolecular properties must, of course, be of a piece with existing theories for systems of small molecules—a condition that is surely obvious. This fundamental principle was central to the Harvard laboratory of Edwin Cohn and John Edsall (where I had been a postdoc) and is emphasized in the title of the book they published in 1943 (Cohn & Edsall, 1943). It was brilliantly exemplified even much earlier in a paper by Kai Linderstrøm-Lang (1924), in which he applied the principles of the Debye-Hückel theory (for the behavior of small ions), only 1 year after it was first published, to the ionization of acidic and basic groups of proteins. (My postdoctoral research project was based on that paper.) This universality meant that there were no brand new principles to be learned. However, I had only a smattering of knowledge about the particular physicochemical properties that were likely to be best suited to advance understanding of protein macromolecules—experimental methods such as ultracentrifugation tend to be of little value for small molecules and had been neglected in my graduate training.

Of course, there were some who understood already. Paul Doty, for example, whose paper on collagen with Helga Boedt-

Reprint requests to: Charles Tanford, Tarlswood, Back Lane, Easingwold, York YO6 3BG, UK.

Charles Tanford received his undergraduate and Ph.D. degrees in chemistry at New York University and Princeton University, respectively. He rose through the ranks to Professor of Chemistry at the University of Iowa from 1948 to 1960; he was Professor of Biochemistry at Duke University from 1960 to 1980, then Professor of Physiology until retirement in 1988. He has devoted his time since then to writing popular books about science and its history. Professor Tanford is a member of the National Academy of Sciences. He has been a visiting professor at Harvard (1966), Eastman Visiting Professor at Oxford University (1977–1978), Walker-Ames Visiting Professor at the University of Washington (1979), and Reilly Lecturer at Notre Dame (1979). He received an Alexander von Humboldt Prize in 1984 and the ASBMB-Merck Award in 1992.

ker (Boedtker & Doty, 1956) is a brilliant piece of authoritative experiment and interpretation. But that didn't help me. I had never derived the pertinent equations and (given my inherent ideas about "responsibility" in research) couldn't base my own research on somebody else's confidence in their validity. My process of self-education was slow; it took the form of writing a textbook.

The book

I began to write *Physical Chemistry of Macromolecules* (Tanford, 1961) not long after I entered my first academic position as Assistant Professor of Physical Chemistry at the University of Iowa. The book was 10 years in the writing; it was not published until after I had left Iowa to join the Biochemistry faculty at Duke University, which I did in 1960.

The book had its roots in a proposition by a group of physical chemistry graduate students at Princeton University to write a multivolume treatise on the totality of physical chemistry. We were all students of (or at least inspired by) Henry Eyring, one of the great theoreticians of the time, who had the knack of being able to think and write about complex concepts in an unpretentious (at times even homespun) way—we hoped that we had managed to absorb some of his skill. We had divided the project among ourselves, as dictated by our special interests: I had been stimulated by Walter Kauzmann to move into protein chemistry and that made it logical that "large molecules" should be my domain. It turned out to be the only part of the project that actually got off the ground, presumably for the simple reason that I had the greatest need for such a book to promote my own research and teaching.

As it turned out, I was greatly stimulated in my efforts by the obvious interest expressed by publishers. American publishers in those days sent their editors out across the country to find budding authors, and Bob Polhemus of John Wiley and Sons turned out to be a particularly congenial advocate—he came to Iowa City once a year and I also met him periodically at Wiley hospitality suites at national scientific meetings. As the book began to take shape, other publishers entered the scene; some of them were ready to write contracts on the spot, but I felt I had a prior commitment to John Wiley. When I discussed the matter with Polhemus, he said that John Wiley, too, would give me a contract then and there—provided that I would be content with their standard royalty rate, which was less than that offered by some of their competitors.

I immediately agreed, and settled down to the writing. Most of it was a thorough reworking of classical theoretical material—Einstein, Stokes, Debye, etc., not specifically directed at macromolecules at all—but I wanted to be sure that I understood everything in depth. Since (at least for me) vigilance tends to slip if one just works one's way step-by-step through some published analysis, my procedure was to look at the assumptions at the beginning of a paper and the result at the end (usually a final equation or several of them) and then to try to go from beginning to end on my own, with as little reference as possible to the original text. Some of the book actually reads as though it were a record of personal discovery, rather than a "reworking," and in the sense I have described, there is some truth to that. It is likely that this method of working things out contributed to the book's ultimate readability, making it easier for the reader to understand the fundamentals that were involved. Toward the end of the process the book did come to include some actual new contributions that I myself made to the subject, notably the work on electrostatic interactions, which I did with J.G. Kirkwood at Yale in a sabbatical year of 1956/1957. (I had a Guggenheim Fellowship to meet most of the cost. They were still an essential lifeline for academic scientists at the time.)

A more indirect motivation for persisting with the book came from the burgeoning industrial polymer industry. Publishers in search of authors were not the only frequent outside visitors to my Iowa City office; agents of American industry came, too, in search of well-trained chemists and most of them showed unrestrained enthusiasm for my graduate students, who were using in their research the kind of laboratory procedures that were basic tools of "polymer chemistry." Here we were, for example, measuring viscosities! How many university laboratories in those days were doing that? And viscosity measurements were the almost universal way of measuring molecular weight in the synthetic polymer industry, indicating the point when polymerization had proceeded to the desired extent and the process should be interrupted. Cheers all around, but for me there was a problem. We weren't using viscosity to measure molecular weight, but something that was loosely called "molecular shape"—"spatial conformation" might be a better word today. For example, catalase and collagen have close to the same molecular weight, but $[\eta]$ in aqueous solution is 3.9 cc/g for the former, whereas Boedtker and Doty measured $[\eta] = 1{,}150$ cc/g for the latter—the interpretation being that collagen molecules are long thin rods, whereas catalase molecules are compact globules. How could this same procedure be measuring molecular weights in an industrial laboratory? I had no idea; it was something I had to work through and figure out. The basic law had to be the same for natural and synthetic molecules. (I don't suppose that any protein chemist today ever uses viscosity as a tool—what it does is to measure "hydrodynamic radius" more precisely than other methods can, but at the cost of what would be prohibitively large amounts of protein by today's standards.)

An aid to my efforts appeared in 1953, with the publication of Paul Flory's book, *Principles of Polymer Chemistry* (Flory, 1953). It was devoted exclusively to the synthetic polymer regime and matters such as the dependence of viscosity of polymer solutions on molecular weight were treated with the utmost clarity and authority. But the exclusivity of its focus meant that the "first" principle for any topic, the point in the logical sequence at which identical equations could still be used for all conceivable kinds of large molecules, received relatively casual treatment, with less detail than I needed. Most of the book was actually based on Flory's own original work; it was colored by the heterogeneity of synthetic polymer preparations with respect to chain length, as contrasted with the fixed number of amino acids in a given protein polypeptide; and also by the microheterogeneity of the flexible conformation of an individual organic polymer chain in the organic solvents where they were normally studied. In short, to adapt it to my own ultimate purpose, I had to "rework" Flory's work just as much as I needed to do for the earlier classical theoretical studies of Stokes, Einstein, etc.

One more influence must be mentioned: I asked for and received critical appraisals of many parts of the book from friends and colleagues. R.L. ("Buzz") Baldwin was especially helpful. I stood somewhat in awe of the analytical ultracentrifuge, both on account of its enormous physical size and its central role in the history of protein chemistry, and had devoted a chapter to

all one could learn from its use. Baldwin summarily demolished that idea, pointing out that the 2 uses of the ultracentrifuge, measuring, respectively, equilibrium and rate of transport, had an entirely different theoretical basis, and that physical theory, not instrumentation, should be the prime focus. His comments led me to a thorough reorganization, in which sedimentation equilibrium was treated in my chapter on thermodynamics, whereas sedimentation rate became part of the chapter devoted to all rates of motion in a viscous fluid.

As I already noted, I had a contract to publish *Physical Chemistry of Macromolecules* with John Wiley and Sons long before the manuscript was finished or even the contents finalized. There has been no peer review; that came only after submission of the final manuscript, and when it came it was a disaster. There were 2 reviewers and their criticism was scathing; I had got it all wrong, they said, and the book was declared effectively unpublishable. The names of the reviewers were of course not revealed, but it was easy to guess who they must have been from the content and style of their criticisms. They were both self-styled experts in protein hydrodynamics, but not in the same league as Baldwin—one of them, for example, had a few years earlier proposed a new way to process hydrodynamic data that in effect demolished the fine discriminatory value of hydrodynamic measurements. I knew their criticisms were invalid.

My publishers of course did not know that and called me on the carpet. No friendly Bob Polhemus to talk to me this time, but a sterner more senior editor, Bill Grimshaw. "You will have to make major changes," he told me. I was of course prepared for that demand. I had brought my copy of the contract with me and laid it on the table. I told Grimshaw that I had every confidence in what I had written and would not change a word. I told him that I realized that it had been a mistake for his company to have given me a contract before a review, that I was reasonably sure that I could find another publisher, and that I would willingly tear up my Wiley contract on the spot. But they had an old-fashioned sense of honor in the publishing business in those days. Grimshaw would not consider reneging on the contract; when he could not argue me into making changes, he reluctantly agreed to publish my text unchanged. And that's what happened.

The book was in fact quite a success and served as a valuable textbook and reference for a whole generation of biochemistry and biophysics students. It only recently went out of print, more than 30 years after publication, but I still receive frequent requests to permit reproduction of parts of the book for distribution to students.

The laboratory

The work done in my laboratory with the aid of students and postdocs was closely linked to (sometimes inseparable from) the subject matter of the book. I have already indicated how some of the topics in the book arose from a need to understand the theoretical basis of experimental methods that we had begun to use in the laboratory. But the converse applied just as often: the gradual expansion of the book to cover all facets of the physical chemistry of large molecules suggested experiments that cried out to be done. There were so many, in fact, that it was possible to pick and choose, to maximize the ratio of perceived benefit to the effort that needed to be expended. The time to get results was a factor, too. I was not cast in the heroic mold of the X-ray crystallographers, who were prepared to struggle for many years with little to show other than Patterson maps and without the certainty that the result they sought was actually achievable. I had need for frequent boosts to my morale in the form of concrete results—not just publications for their own sake (to be counted, bolster my c.v. and impress my elders), but actual creative pieces of work that, at least in my own eyes, advanced the frontiers of knowledge a little, even though my contemporaries might not value the particular advance that was being made.

The most important of our laboratory projects arose directly from my now clear perception of the huge conformational difference between coiled synthetic polymers (normally in organic solvents) and the common native proteins in aqueous salt solutions. Could conditions be found under which the distinction disappears, where the interactions that normally hold protein molecules into their compact structures could be broken, where proteins would behave just like polymer coils? It seemed to me that an investigation of this question was essential to solidify the macromolecular concept. It was absurd to think (as I stated above) that macromolecules and small molecules of similar composition might be subject to different laws of physics. A *fundamental* difference between synthetic and natural polymeric molecules would be equally absurd and it seemed essential to me (almost an obligation) to demonstrate that no such absurdity prevailed.

There was evidence from a variety of sources that concentrated guanidinium chloride (one of many known protein "denaturants") might provide a suitable medium, and this proved to be the case. Our study was thorough and was led by 2 visitors with previous experience in synthetic polymers, Kazuo Kawahara from Japan and Savo Lapanje from the University of Ljubljana; my long-time associate Yas Nozaki was also a member of the group. Intrinsic viscosity indeed became a strict function of chain length (n) in the guanidine solvent: the equation $[\eta] = 0.68n^{0.67}$ applied to data for molecular weights ranging from 3,000 to 200,000, independently of the native function of a protein—our list included even myosin, which in the native state is not in the compact globular category at all. We measured osmotic pressures, focussing on the *concentration dependence*, which is theoretically related to the volume in solution from which 1 molecule excludes its neighbor and was therefore a favorite tool of the polymer chemist for determining molecular dimensions—there were ferocious experimental problems in using the corrosive guanidinium solutions in an apparatus designed for benign organic solvents, but they were overcome by the consummate skill of Savo Lapanje. We showed also that spectral properties in guanidine became additive functions of amino acid composition of a protein. Nozaki demonstrated the same for acid–base equilibria, showing that all the effects of electrostatic interactions etc., which affect such equilibria in the native state, had disappeared.

Some of the proteins we used had disulfide bonds, which of course had to be reduced before measurements were made because covalent crosslinks would have prevented free expansion into an otherwise unrestricted coil. We also insisted that consistency with coil behavior or with properties of amino acids and small peptides in the same solvent had to be quantitative: acid–base dissociation constants, for example, were *exactly* equal to those for the same groups on compounds of low molecular weight,

as remeasured in the same guanidine solvent. The coefficient 0.67 in the exponent of the viscosity equation was in the expected range for a polymer in a "good" solvent, i.e., one that penetrates avidly into all parts of the molecular coil. In much later work, after my collaboration with Jacqueline Reynolds had begun, we studied proteins that had been denatured by the detergent sodium dodecyl sulfate. Once again a kind of universal conformational state was attained, in which properties such as viscosity became regular functions of molecular weight, but there was a large quantitative difference, e.g., the exponential coefficient in the viscosity equation was 1.2 instead of 0.67. The denatured state in the detergent is rodlike and not loosely coiled, a conclusion supported by numerous other techniques.

The hydrophobic "bond"

A parallel investigation (chronologically actually earlier than the one I have just discussed) was into the question of what the forces are that hold proteins into specific tight structures. It was appropriate to use the word "noncovalent bond" in relation to this question: specifically, hydrogen bonds were favorite candidates. Hydrogen bonds were already accepted in protein chemistry as the essential elements of Pauling's α-helix and β-pleated sheet, but these polypeptide backbone structures by themselves could not account for the formation of the tight little molecular balls that many common proteins were known to be. What more natural than to invoke more hydrogen bonds, between amino side chains this time, e.g., tyrosyl or arginyl and carboxylate groups? I was one of many who (in 1953) flirted with this possibility. Harold Scheraga and his colleagues were more enthusiastic advocates. In extensive publications they ascribed many anomalies of protein behavior to the making and breaking of such bonds. However, quantitation of the phenomena to be explained required that an energy (or enthalpy) of about 6 kcal/mol be assigned to hydrogen bonds, a value that is appropriate for hydrogen bonds in a vacuum, but not in aqueous solution, where ΔH is actually close to zero—Irving Klotz has explained this with particular clarity in his own "recollections" in this journal (Klotz, 1993). Scheraga, for reasons that are not clear, ignored this difficulty and continued to promote side chain hydrogen bonds for many years: he even went so far as to put a huge amount of effort into constructing a hypothetical atomic model for the structure of ribonuclease, based on maximization of the number of such bonds (Scheraga, 1960). Needless to say, the publication of the first X-ray structure of a protein, shortly thereafter, put an end to this line of thinking.

In the meantime Walter Kauzmann (1954) had made a radically different proposal, which he called "hydrophobic bond"—based on hydrogen bonds in the solvent (H_2O) and between the solvent and charged or polar protein groups, which left nonpolar groups of a protein clinging tightly to each other in the molecular core. The idea was not new and had in fact been central dogma for detergent chemists for many years (Hartley, 1936). But it was new to protein chemists and a revelation to me. I spent the year 1956/1957 at Yale University, developing under the guidance of Jack Kirkwood a comprehensive theory for the electrostatic interaction between charged groups of proteins, now confidently assigned, freely hydrated, to positions at (or projecting just beyond) the smoothed surface that one customarily imagined as the interface between macromolecule and solvent. This work indirectly supported the hydrophobic effect as the primary force that directs the final stage ("tertiary structure") in protein folding, as I explained in a review of the general problem of protein conformation at a symposium held in Paris—the proceedings of which (Neuberger, 1957), incidentally, provide a fascinating historical record of protein chemistry still in considerable flux!

Kauzmann himself published the definitive review on the hydrophobic bond in 1959 (Kauzmann, 1959); some of the experimental work in my own laboratory for several years thereafter was devoted to buttressing the concept further. Kauzmann's 1959 review is often cited as if it were his initial paper on the subject and I have specified exact dates here to set the record straight. His 1954 paper was explicit and unambiguous—he was many years ahead of the rest of us, who may nowadays be loosely grouped together as among the originators of the hydrophobic hypothesis.

Concluding thoughts

"Books do furnish a room," as one of our contemporary literati has stressed (Powell, 1971). Do they have a place in the laboratory as well, in spite of their slow gestation periods when compared to the rapid pace of research itself? Or are rapid publications all we really need? I believe that books do in fact play a more important role than is popularly acknowledged. No one can work in an intellectual vacuum. Focussed research had to have its roots in an accepted body of knowledge: in this case the assumption that proteins are truly macromolecular was crucial to methodology and interpretation. My book and (more generally) all of us who were on the "sidelines" served to reinforce this basic axiom repeatedly. Proteins were true macromolecules with stable primary bonds; each could undergo transitions (usually reversibly) among many different well-definable conformational states; the native state under physiological conditions seemed at least very similar to the one in protein crystals, etc. Would the crystallographers have persisted so faithfully without our implicit support? The glory and the laurels in the history of protein chemistry have rightly gone to those who set themselves goals that were far beyond the mapped territory of the time and who persisted against improbable odds to attain those goals. But it seems to me that my book and other items I have cited here were not irrelevant.

As I mentioned earlier, I did not at the time see my own work as limited to providing this kind of "background." I was motivated in part by the notion that our laboratory studies were contributing to a body of knowledge on the basis of which actual protein structures could begin to be imagined. As actual X-ray structures began to multiply, this motivation was lost and my interest turned to other matters: antibodies, cell membranes, etc., not directly related to the nitty gritty of the physical chemistry of macromolecules in solution. It is amusing therefore to note a recent revival of quantitative physicochemical studies of proteins in solution, in relation to changes in molecular conformation (sometimes akin to reversible denaturation) that are an essential part of the biological function of many protein molecules that are not of the crystallizable variety. As one commentator (Gratzer, 1987) has put it: "The whine of the Model E (ultracentrifuge) is once again to be heard in the land, like the voice of the turtle, to gladden the hearts of the old-timers."

References

Boedtker H, Doty P. 1956. The native and denatured states of soluble collagen. *J Am Chem Soc 78*:4267–4280.

Cohn EJ, Edsall JT. 1943. *Proteins, amino acids and peptides as ions and dipolar ions*. New York: Reinhold Publishing.

Flory PJ. 1953. *Principles of polymer chemistry*. Ithaca, New York: Cornell University Press.

Gratzer W. 1987. Protean proteins perceived. *Nature 328*:669–670.

Hartley GS. 1936. *Aqueous solutions of paraffin-chain salts*. Paris: Hermann & Cie.

Kauzmann W. 1954. Denaturation of proteins and enzymes. In: McElroy WD, Glass B, eds. *The mechanism of enzyme action*. Baltimore: Johns Hopkins Press, pp 70–110.

Kauzmann W. 1959. Some factors in the interpretation of protein denaturation. *Adv Protein Chem 14*:1–63.

Klotz IM. 1993. Solvent water and protein behavior: View through a retroscope. *Protein Sci 2*:1992–1999.

Linderstrøm-Lang K. 1924. On the ionization of proteins. *C R Trav Lab Carlsberg 12*:73–98.

Neuberger A, ed. 1957. *Symposium on protein structure*. London: Methuen & Co.

Powell A. 1971. *Books do furnish a room*. London: Wm. Heinemann.

Scheraga HA. 1960. Structural studies of ribonuclease. III. A model for the secondary and tertiary structure. *J Am Chem Soc 82*:3847–3852.

Tanford C. 1961. *Physical chemistry of macromolecules*. New York: John Wiley.