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HISTORY

How protein chemists learned about the hydrophobic factor

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Abstract

It is generally accepted today that the hydrophobic force is the dominant energetic factor that leads to the folding of polypeptide chains into compact globular entities. This principle was first explicitly introduced to protein chemists in 1938 by Irving Langmuir, past master in the application of hydrophobicity to other problems, and was enthusiastically endorsed by J.D. Bernal. But both proposal and endorsement came in the course of a debate about a quite different structural principle, the so-called "cyclol hypothesis" proposed by D. Wrinch, which soon proved to be theoretically and experimentally unsupportable. Being a more tangible idea, directly expressed in structural terms, the cyclol hypothesis received more attention than the hydrophobic principle and the latter never actually entered the mainstream of protein science until 1959, when it was thrust into the limelight in a lucid review by W. Kauzmann. A theoretical paper by H.S. Frank and M. Evans, not itself related to protein folding, probably played a major role in the acceptance of the hydrophobicity concept by protein chemists because it provided a crude but tangible picture of the origin of hydrophobicity per se in terms of water structure.

Keywords: cohesive forces in biology; cyclol hypothesis; hydrophobic effect; protein folding

Protein molecules are long chain polymers of amino acids with often hundreds of monomers strung along a line. Yet physical measurements indicate that most of the readily soluble proteins exist in solution, not as long extended chains, but as tightly packed little entities, almost spherical in shape. Many of these proteins can be crystallized, with retention of the same compact conformations. This raises the question: what is the force that collapses the chains into globules?

It is now generally agreed that the hydrophobic force is the crucial component of the several factors that contribute to the overall energetics of collapse and most elder statesmen among today's protein chemists would without hesitation ascribe their first insight into this vital notion to a review written by Walter Kauzmann (1959), following an earlier statement (Kauzmann, 1954), which did not reach as wide an audience. John Edsall (1992), for example, writing about the early days of his career, mentions early studies on hydrophobic effects in general (data for various organic solutes) but points out that the first clear look at these phenomena in their relation to protein structure "did not come until 1959, in Walter Kauzmann's great paper in vol. 14 of *Advances in Protein Chemistry*." Similar credit is given by younger reviewers who cannot rely on personal recollections, e.g., Rose and Wolfenden (1993) in a recent comprehensive article cite the "dramatic change" toward the hydrophobic point of view and ascribe it to Kauzmann's "seminal review."

However, though Kauzmann cites no earlier sources in his publications, he today disclaims originality for the proposal per se, recalling that the idea had been "in the air" before then (Kauzmann, pers. comm.). Since the concept has become so widely used, not only for structural containment, but also in related contexts, as part of the explanation for the specific biological functions of proteins as enzymes, antibodies, carriers, etc., it seems important to trace its true origin and that is the purpose of the present paper.

The result of the investigation will be to show that the principle was in fact first clearly stated in 1938 by Irving Langmuir, a complete outsider to the protein community, but (in his own field of surface chemistry) one of the great chemists of all time. The first person with a primary interest in proteins to become a hydrophobic enthusiast was the crystallographer John D. Bernal, who got the idea directly from Langmuir. What may be of special historical interest is that this conceptual transfer was actually a side-show, peripheral to an acrimonious dispute between Langmuir and Bernal regarding a bizarre chemical theory, the "cyclol hypothesis." The emphasis was on cyclols; hydrophobicity was inserted into the argument as a weapon in the debate.

Definition and application in other areas of chemistry

The word "hydrophobic" has been in use for hundreds of years to describe the condition of persons afflicted by rabies ("hydropho-

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Irving Langmuir and J.D. Bernal discuss viruses at a meeting of the British Association in Nottingham in 1937. Dorothy Crowfoot is looking on. (From Crowfoot-Hodgkin, 1980.)

bia"). Its use as a technical term in chemistry, to denote antipathy between water and many kinds of apolar chemical entities, is about a hundred years old. It is particularly useful in the kind of situation treated in this paper, where the hydrophobic antipathy does not involve an entire solute molecule but only a part of it, a moiety (usually hydrocarbon) on an amphiphilic organic molecule, one that contains both hydrophilic and hydrophobic domains. A purely hydrophobic particle would tend to have low solubility in water, with little likelihood of interesting consequences. The dichotomous affinities of an amphiphilic particle, on the other hand, with some parts attracted to water and others repelled, can produce molecular orientation or aggregation or otherwise unexpected conformational contortions.

Ever since hydrogen bonds (Latimer & Rodebush, 1920) and their relation to the properties of water (Bernal & Fowler, 1933) were first recognized, it has been understood that hydrophobic antipathy is a simple consequence of the great strength of hydrogen bonds in liquid water. Contact between apolar molecular surfaces and water is avoided, not because there is actual repulsion between them, but because the strong attraction of water molecules for one another dominates the scene: in the case of hydrocarbon, for example, both hydrocarbon-hydrocarbon or hydrocarbon-water attractions are weaker in comparison. In any application where hydrocarbon entities are forced into close proximity, it is essential to appreciate the distinction in this definition between the hydrophobic force—originating in the surrounding solvent—and "like to like" attraction (preferential van der Waals forces).

The first use of the hydrophobic concept as a factor in molecular orientation was made in relation to surface tension, by the German physical chemist Isidor Traube (1891; see biography by Edsall, 1985). His results showed that many organic solutes are adsorbed at a water/air interface, with the polar ends of molecules in the water and nonpolar parts sticking out. Irving Langmuir subsequently became the true master analyst of events at this interface: his 1917 paper on surface layers of organic molecules (Langmuir, 1917) is a tour-de-force and was the basis for award of the Nobel Prize for chemistry in 1932. Langmuir displayed an extraordinary pictorial molecular imagination, by which he was able to interpret measurements of the surface areas as a function of surface pressure so as to visualize in his mind (given that he knew which end of an adsorbed molecule was in the water and which stuck out) exactly what individual molecules in the film were doing. He recognized the permanent "kink" caused by a double bond in an aliphatic hydrocarbon chain, to give just one example. None of his conclusions has ever been seriously challenged.

Another early prominent field of application is to soap micelles in aqueous solution, where we are dealing with molecular aggregation of amphiphilic monomers. The treatise of G.S. Hartley (1936) on this subject is particularly articulate in stressing the distinction between the hydrophobic mechanism for aggregation and "like to like" attraction, a mechanism earlier invoked by McBain and Salmon (1920).

What is now seen as the most obvious biochemical application of the hydrophobic idea, much simpler than extension to soluble proteins, is to biological lipids and cell membranes. The amphiphiles here have very large hydrophobic domains and exist as bilayers (which can be thought of as extended micelles), with the hydrocarbon parts facing each other in the middle. The famous paper by Gorter and Grendel (1925) on this subject was explicitly derived from Langmuir's 1917 paper, with compelling experimental data in support. It is now considered a great classic in the field of biology, but at the time it was ignored. Gorter himself, whose active career continued for many years despite crippling arthritis, did not push it, partly because he was primarily a physician and pure science was only a secondary interest, but undoubtedly also because of the hostile reception of his work. I have described this ludicrously slow acceptance of the bilayer concept for cell membranes (which took more than 40 years) in another place (Tanford, 1989).

It is worth noting that the seminal work on micelles or bilayers that I have cited probably had no *direct* influence on the exploration of theoretical causes by protein chemists. Extension to proteins in not obvious. Most people at the time would still have pictured a "like to like" mechanism as an important factor in hydrocarbon segregation. In micelle or bilayer formation, where one is dealing with solute molecules that are homogeneous or nearly so, with *very long* aliphatic hydrocarbon chains, this concept (perhaps lining up in parallel) has some intrinsic appeal. In applying the idea to proteins, "like to like" lacks any such plausibility: the non-polar moieties of protein amino acid side chains are not only short in length, but some are aliphatic and some aromatic. Furthermore, they are all mixed up with polar groups along the polypeptide chain, rather than neatly segregated.

State of knowledge of protein structure in the 1930's

There was intense interest in protein chemistry because proteins were seen to control a huge variety of biological processes: enzymatic activity, antibody specificity, oxygen binding, and even genetics and inheritance. Most people still thought that proteins were the carriers of genetic information! (Judson, 1979). Avery's definitive paper that proved it was DNA was only published in 1944 (Avery et al., 1944), and many people were not finally convinced until the "Waring Blender" experiments of Hershey and Chase (1952).

Published symposia (Protein Chemistry, 1938) and a famous book (Cohn & Edsall, 1943) mirror the state of knowledge about proteins—the book, though published in 1943, has its roots in the 1930's with copious references to that decade. These sources show that it was established that proteins consist of long chains of amino

acids in peptide linkage and that free amino and carboxyl groups carry actual ionic charges at neutral pH (and that these groups in proteins behave normally in response to changes in pH, much as they do in amino acids and other small molecules). Molecular weights were known for many proteins—often (e.g., for hemoglobin) quite accurate in the light of modern definitive values. The distinction between "fibrous" and "globular" proteins was established and a considerable number of the latter were being obtained in crystalline form. It was understood both from physical measurements in solution and from crystallographic results that the globular proteins were folded tightly into small compact particles. The phenomenon of protein "denaturation" was known and fitted into this picture as an "unfolding" of the tight globular structure.

Great hopes were pinned on the possibility of obtaining unambiguous structural data for crystalline globular proteins (Bernal et al., 1938; Edsall, 1943) but tremendous practical hurdles had first to be overcome and most protein chemists would probably not have believed that it could happen as quickly as it did.

For this reason simple structural generalizations were welcomed and given more credence and publicity than they merited. One such item came from The Svedberg, one of the great figures in the history of protein chemistry, who developed both the theory of ultracentrifugation and the building of the first instrument and its exploitation. It was he who first showed that protein molecules of a single chemical or biological specificity were essentially homogeneous with respect to molecular weight, demolishing theories that were still rife at the time that proteins might be colloidal aggregates of smaller units, heterogeneous with respect to molecular weight in the aggregated state. However, Svedberg (1930) still clung to some extent to the idea of small units built into larger ones and proposed from his initial results that all proteins might have molecular weights that were integral multiples of some smaller number (17,000 or 34,000). The best place to catch the flavor of the Swedish enthusiasm for this idea is in the treatise by Svedberg and Pedersen (1940), written after enthusiasm elsewhere was pretty well dead.

Even more intriguing was the hypothesis of Bergmann and Niemann (1937), based in part on Svedberg's proposal and on amino acid content data, that the total number of residues of amino acids in any protein is expressible by the formula $2^m \times 3^n$, where m and n are integers and that the number of residues of any individual amino acid was expressible by the formula $2^{m'} \times 2^{n'}$ where m′ and n′ could be integers or zero. Now here was a "magic formula," which, if true, could lead to all kinds of mathematical speculation as to how proteins might be assembled in the living cell! But, of course, it wasn't true.

It is important to appreciate that such wild ideas were given every full consideration and not discarded out of hand so that we can understand why the cyclol hypothesis (see below), which had no basis in fact at all, was also seriously considered before being soundly rejected.

The problem of protein folding: Hydrogen bond theory

The existence of the intrinsic problem about globular proteins, which I have already defined, was broadly recognized. The peptide bond alone should produce a macromolecule that ought to be a long and flexible chain, which a globular protein certainly is not.

A uniquely lucid paper on the subject came from China as early as 1931, from the laboratory of Hsien Wu (1931; see Edsall, 1995). Its main topic is protein denaturation, which Wu sees in an essentially modern context of protein structure—the native protein is an organized compact molecule; denaturation destroys organization, normally without change in molecular weight, often reversibly. The native molecule can be thought of as a sort of submicroscopic crystal, held together primarily by non-covalent interactions. What are these interactions? Wu assumes without feeling a need for justification that the force of attraction (in a single molecule) is between *polar groups*, similar to the force that holds many molecules together in a protein crystal. Hydrogen bonds were not yet widely known. Had they been, Wu would undoubtedly have considered them a likely possibility.

Hydrogen bonds in fact became well known later in the 1930s, partly through the seminal paper on water structure by Bernal and Fowler (1933)—though it might be noted that these authors initially avoided the use of the word "bond" in relation to the phenomenon. The idea of shared electrons bonding atoms together was still fairly new and conservatives were understandably reluctant to extend the meaning of the term (Pimentel & McClennan, 1960).

A likely specific role for hydrogen bonds in the maintenance of native protein structure was first stressed with great confidence by Mirsky and Pauling (1936), in a paper that (like Wu's) addressed itself to "denaturation," and hydrogen bonds soon became part of the fashionable dogma. A hydrogen bond theory for what holds globular proteins together is intuitively attractive, being a *direct* cohesive mechanism, in contrast to the concept of "hydrophobicity," where the source of the force is outside the protein molecule per se.

The cyclol theory (Dorothy Wrinch)

A very different kind of hypothesis, the "cyclol" hypothesis, was proposed by Dorothy Wrinch, an English mathematician, who (among other attachments) was at one time infatuated with Bertrand Russell and his philosophy. She had no formal training in chemistry and at best a rudimentary comprehension of the rules of evidence in scientific research. She was arrogant and felt persecuted when criticized, but in retrospect her miseries seem self-inflicted. A full account of her tempestuous life is available (Abir-Am, 1987).

Wrinch's proposal was a geometrical construct requiring rejection of the simple peptide link between amino acid residues of the protein backbone. It was replaced (by means of a chemical change analogous to lactam–lactim tautomerism in other organic compounds) by a three-pronged arrangement which lends itself to construction of six-membered rings and honeycomb-like polyhedra. A barrage of papers appeared (e.g., Wrinch, 1936a, 1936b, 1937a, 1937b), claiming not only to account for molecular compactness, but also to explain the supposed molecular weight classes suggested by Svedberg. (See Wrinch, 1938, for a review lacking the tone of urgency of her shorter papers.) It was sufficiently sensational to give Wrinch a hearing all around the world, but it did not take much time before it was found to be all wrong—both thermodynamically and sterically inadmissible. Linus Pauling, in a private report to Warren Weaver of the Rockefeller Foundation (Hager, 1995, p. 227), based on a personal meeting with Wrinch, even labeled her papers as "dishonest." The whole matter might well be dismissed as a footnote, were it not that the cyclol controversy became an accidental vehicle

for introduction of hydrophobicity into the protein chemist's vocabulary.[1]

Irving Langmuir gets involved

Following her cyclol proposal, Wrinch began to bombard famous people with letters, seeking interviews and collaboration. Linus Pauling was her target in the summer of 1936, for example, but no meeting between them resulted until 1938 (Hager, 1995). She was more successful with Irving Langmuir, another giant in the history of chemistry, famous both for his industrial research (the originator of the incandescent light bulb in its present design) and for his more basic work in surface chemistry, in which hydrophobicity played a paramount role, as described earlier in this paper.

Langmuir may have been receptive to Wrinch's approach because she not merely sought approval from him, but suggested new experiments, adaptation of his surface balance measurements to protein layers, firing his imagination with a promise of exciting new vistas for exploration. In any case, Langmuir asked Wrinch to visit him in Schenectady in December 1936 and initial experiments with the surface balance were done and were successful. "This should have great value as a biological tool: very likely it will find a place in the diagnosis of disease," he said in a lecture (Langmuir, 1937). Surface studies of proteins did, in fact, attract a small number of workers but had only a minor impact and none at all on medical diagnosis.

The first paper resulting from this collaboration appeared almost at once (Langmuir et al., 1937) and was experimentally oriented—a detailed and persuasive description of the multi-layer method previously devised in Langmuir's laboratory by Katherine Blodgett and its likely advantages for study of protein monolayers. (The protein in these experiments was laid down on top of multiple layers of barium stearate.) Only the final sentence of the paper presented a jarring tone, advocating the unsupported claim that "the protein monolayer is a two-dimensional network held together by strong elastic springs" and that the early results are "not in accord with a structure consisting of polypeptide chains." Subsequent papers failed to maintain the high ground of the initial work and degenerated quickly to polemic, supported by little or only superficial experimental evidence.

This involvement of Langmuir with Dorothy Wrinch is what led to the first firm proposal of the modern thermodynamic argument for the importance of hydrophobicity. Langmuir reached back into his past experience, to the work at the air/water interface that we have already mentioned. He recognized that a substantial fraction of any protein's side chains are hydrophobic and that the need to remove these entities from contact with solvent must be a driving force for the folding of polypeptide chains into the compact "globules" that the soluble protein molecules were even then unequivocally known to be. In one of the papers (Langmuir & Wrinch, 1939) Langmuir goes so far as to make a quantitative estimate: stabilization of 2 kcal/mol will result when 2 CH_2 groups combine with each other instead of with water. All this was said in the context of seeking support for the cyclol hypothesis—i.e., essentially arguing that, given the need to "collapse" into a small space, the polar groups of a polypeptide must be willing to accommodate themselves in a manner that might otherwise not seem natural—but the generality of the argument for collapse itself was not lost thereby.

In retrospect, it is actually difficult to understand how Langmuir could have remained an advocate for the cyclol proposal once he had examined it in detail. Wrinch's structures were sterically impossible (Pauling & Niemann, 1939). In deducing the geometry of the structures Wrinch had focused on the polypeptide backbone, with amino acid side chains represented simply by the letter "R." When "R" was replaced by actual atoms, there turned out to be not enough room for them! In one example, close to Langmuir's own special interests, Wrinch explicitly suggested cyclol patterns for adsorbed protein surface layers, which Neurath and Bull (1938) easily demolished on this basis—Wrinch models tolerate an average of 10–17 $Å^2$ per residue, depending on which of several suggested cyclol structures is used. Actual protein R groups average about 20–25 $Å^2$. How could Langmuir, with his demonstrated exquisite pictorial imagination and his sense of how molecules occupy space, have been taken in?[2]

Some weeks of fierce debate: J.D. Bernal and others take up the challenge

Langmuir (1938a) first spelled out the hydrophobic theory for protein folding at an exceptionally lively Cold Spring Harbor symposium on protein chemistry in the summer of 1938. His paper was mostly devoted to protein monolayers but the implication with respect to globular proteins was made quite clear. Other papers at the symposium dealing with globular proteins (including two explicitly directed at denaturation) should certainly have made reference to hydrophobicity if they knew about it but none did. Danielli (1938), who spoke about protein films at an oil/water interface, recognized the hydrophobic/hydrophilic dichotomy but did not connect it to the problem of protein folding. Neither Langmuir nor Danielli appear to have struck a responsive cord among the assembled protein chemists. For example, Edwin Cohn, John Edsall, and some of their associates were among the participants, but there is no mention of the topic in the treatise (Cohn & Edsall, 1943), which was then in preparation. (On the other hand, they devote three pages to a description of Wrinch's cyclol hypothesis!)

Things became quite different later in the year in England, where Irving Langmuir's endorsement of Wrinch's hypothesis triggered a fierce debate. John D. Bernal (Crowfoot-Hodgkin, 1980) was the intellectual leader. An Irishman, born on a farm near Tipperary, he was twenty years younger than Langmuir but already recognized as no less a genius. He was a theoretical physical chemist by training and co-author of the famous paper on water structure that has already been cited. By 1937 he had entered the periphery of the protein research community by setting out deliberately to determine the structure of proteins by means of X-ray crystallography. Moreover, unlike William Astbury, who had been working on the

[1]There was no anti-feminine prejudice involved in the criticism of Wrinch's work. On the contrary, some of her adversaries (including Bernal) were roused to action by their admiration for the work of Dorothy Crowfoot, a Bernal student and subsequently a Nobel laureate. Wrinch had taken unconscionable liberties with Crowfoot's data in an attempt to bolster her geometrical fantasies (see below.) Forty years later Crowfoot herself (now Dorothy Hodgkin) retained no ill feeling and wrote a kind obituary, stressing Wrinch's positive influences (Hodgkin & Jeffreys, 1976). She mentions the 1938 Cold Spring Harbor symposium, where Wrinch "captivated everyone with her enthusiasm."

[2]Langmuir's biographer (Rosenfeld, 1966) provides no clues, doesn't even mention Wrinch other than as a co-author in references. It should be noted, however, that he was not granted full access to Langmuir's personal diaries—only selected excerpts were provided. There is thus room for future investigation of the question of Langmuir's motivation.

X-ray diffraction of fibrous proteins, Bernal focused on the globular proteins, the only ones to form true crystals. He had a missionary zeal and is in fact the model for the character Constantine in C.P. Snow's novel, *The Search* (Snow, 1934), who had great ambitions to make discoveries in protein chemistry and to found a National Institute for Biophysical Research—remarkably visionary for the early 1930's.

While almost everyone in the protein community was made angry by the cyclol hypothesis and the uncritical manner in which it was touted, Bernal, who had initially befriended and encouraged Wrinch, clearly felt more personally challenged than anyone else, for the problem so casually and often arrogantly addressed by Wrinch—the intimate arrangement of atoms within protein molecules—was the problem to which Bernal was hoping to devote a lifetime of research. He was particularly angered by Wrinch's claim (Wrinch, 1937c) that preliminary X-ray data for insulin, published by Bernal's Ph.D. student Dorothy Crowfoot (1938), supported the cyclol structure—the analysis that led to this assertion was at best sloppy and incompetent, at worst dishonest (Bernal, 1939a).

The weeks of fierce debate (all of which took place in London) may conveniently be said to begin at a meeting of the Royal Society on protein molecules on November 17, 1938, with T. Svedberg as principal speaker (Discussion on protein molecules, 1939). Dorothy Wrinch crusaded on behalf of her structural theory, which was severely criticized on the spot by the distinguished biochemist A. Neuberger (1939). His criticism was purely chemical: improbability of the postulated tautomeric transition. (At the same time Neuberger virtually demolished the Bergmann-Niemann frequency hypothesis on statistical grounds.) Bernal gave a talk about X-ray crystallography at this meeting but was not yet involved in the controversy.

But only two weeks later, on December 1, Bernal became involved. The occasion was his inaugural lecture as professor of physics at Birkbeck College. It was a general lecture entitled, "The structure of solids as a link between physics and chemistry," but there is anecdotal information (Crowfoot-Hodgkin, 1980) to indicate that his mind was very much on proteins. It appears that the lecture was hastily prepared; slides for it were gathered at the last minute. Bernal went to the Royal Institution to borrow some extra slides from J.M. Robertson but he was collared there by Langmuir (preparing for his lecture the following week?) who attempted to convince him of the merits of the cyclol theory. Bernal was incredulous at his naiveté and presumably tried to argue with him for he was detained for hours and almost late for his Birkbeck lecture.

Next came two lectures by Irving Langmuir, the Pilgrim Trust Lecture at the Royal Society on December 8 and a lecture at the Royal Institution on December 9—both were published without delay (Langmuir, 1938b, 1939). Langmuir came out explicitly with the hydrophobic/hydrophilic principle as a basis for globular protein structure, noted the parallel between globular proteins and soap micelles, etc.—it is a scholarly presentation, though Langmuir of course also reiterated his advocacy of the cyclol theory. If we include the American symposium lecture cited earlier, this adds up to three advocacies in the space of less than six months—these lectures should presumably be regarded as the actual point of entry of the hydrophobic hypothesis for protein folding into the general literature.

During the same period there was a deluge of letters and articles on the cyclol hypothesis in the weekly magazine *Nature*. One issue alone (January 14, 1939) had a paper by Langmuir and Wrinch (1939), which reasserts that the cyclol hypothesis is "confirmed" by the insulin X-ray data, and three letters attacking that assertion, the most scathing by Bernal (1939a), but another almost as strong by Nobel-prize winning X-ray crystallographer Lawrence Bragg (1939). Letters to *Nature* were at that time published within a week or two of receipt and were thus a forum for extremely rapid dialogue, i.e., these letters were all written after the foregoing lectures. The series of communications to *Nature* contains only a single voice of support for Wrinch, a letter by E.H. Neville (1938) of the University of Reading, which ends with the familiar call often heard in support of ideas that lack a foundation: Let anyone who wants to argue with Wrinch propose a better structure than hers.[3]

Soon after, not actually ending the cyclol debate but most important in the present context, is a lecture by Bernal (1939b) at the Royal Institution (January 27, 1939), entitled "Structure of Proteins." In it Bernal has surprisingly been converted, become an advocate himself of the hydrophobic principle. (Though not of course of the despised cyclol theory. In Bernal's own words: "Langmuir has used this picture as a justification of the cyclol cage hypothesis, but it is strictly quite independent of it.")

It is a fascinating phenomenon. In most people's minds an angry rejection of the cyclol hypothesis would probably lead to automatic rejection of everything associated with it, but Bernal managed to find the gem of truth within the dross. He has thought about and been convinced by the hydrophobic mechanism, which had been advocated by Langmuir in the very same paper that Bernal castigated two weeks earlier. The words he now uses are his own, not mere reiterations of Langmuir's, to whom, of course, proper credit is given. He explicitly makes a point that Langmuir does not: "Ionic bonds are plainly out of the question, as they would certainly hydrate," putting down the likelihood of polar links as a mechanism for folding. (I mentioned hydrogen bonds earlier. Ionic "salt links" were also often suggested.) Other direct quotations from Bernal's lecture are: "The behavior of the hydrophobe groups of the protein must be such as to hold it together." "In this way a force of association is provided which is not so much that of attraction between hydrophobe groups, which is always weak, but that of repulsion of the groups out of the water medium."

Bernal of course also discussed X-ray diffraction and the structural information derived from it in the same paper, and ultracentrifuge studies and the like as well. This is a pioneering and prophetic paper, a glimpse into a rare moment when one man's insight was able to encompass simultaneously all the strands of a complex problem, much of which the rest of the protein community would not understand for another twenty years. The paper was reprinted in *Nature* in April 1939 with only minor changes. It was thereby made available to the world at large and many readers (or listeners at the lecture) must have absorbed and stored away the gist of it. I have been able to find no further explicit endorsements in print but it is plausible that the idea was henceforth "in the air," as Kauzmann claimed, mentioned in lectures (Laidler & Meiser, 1995) or informal discussion.

It is worth noting that Bernal's perception is in marked contrast with that of Linus Pauling, who (with Carl Niemann) wrote a paper critical of the cyclol hypothesis some time after the above. This

[3] It turns out (Abir-Am, 1987) that Neville was Wrinch's "intimate friend" and "adoring lover," so his status as an objective outsider is questionable. Later, in the 1950's, Neville proposed marriage to Wrinch, but her ardor had evaporated. It would have been her third marriage.

paper (Pauling & Niemann, 1939) is considered by many as the most devastating of all, which it may well have been by virtue of Pauling's prestige, though much of their evidence was not new. They referred to X-ray data, steric impossibilities, and thermodynamic arguments. In the last connection, they explicitly cite "the stabilizing effect of the coalescence of hydrophobic groups" and even give Langmuir's estimated numerical value of 2 kcal/mol per pair of CH_2 groups. But the number was quoted only to show that it is not large enough to overcome the intrinsic improbability of the cyclol tautomerism per se, without recognition of the more general implications with regard to the stability of globular proteins. The word "hydrophobic" is used here as equivalent to "nonpolar," the force of adhesion between CH_2 groups is equated with the van der Waals force, though the figure given is too large for that. Pauling continued for many years to think of direct intramolecular hydrogen bonds as the only conceivable force for protein folding (see below).

Intervention of the war

1939 saw the beginning of the war in Europe and the effective end of this debate about the underlying cause of protein structural compactness.

John Bernal was a communist and a leading anti-war activist, but nevertheless became Lord Louis Mountbatten's trusted advisor on technical problems—in particular, problems related to the Allied invasion of Normandy in 1944. He was also the principal technical expert on project HABBAKUK, the project to construct huge unsinkable aircraft carriers from a mixture of woodpulp and ice. Bernal accompanied Mountbatten to India and Sri Lanka when the theatre of war moved east after the Normandy invasion; Mountbatten became very fond of Bernal and wrote an appreciation of him for inclusion in Dorothy Crowfoot's biography (Crowfoot-Hodgkin, 1980).

Irving Langmuir was old enough to have seen war service in the First World War and was again eagerly recruited in World War II, when he worked on smoke filters for gas masks, smoke generators to obscure targets from attacking aircraft, and de-icing of aircraft surfaces. The last work led after the war to projects on weather and cloud seeding—Langmuir never returned to the study of proteins.

Linus Pauling, too, was enthusiastically involved with war work, e.g., he invented devices for oxygen analysis in submarines. He was invited to join the atomic bomb project but he declined, for purely selfish reasons ("not because I felt that it was wrong to work on the development of nuclear weapons"). Pauling's crusade against nuclear weapons and the questioning of his loyalty came only later, during and after the McCarthy era.

The younger generation who were to enter the field subsequently were (in America at least) all recruited for war work. Kauzmann, for example, received his Ph.D. in 1940 and became intrigued by proteins in the course of his thesis work on optical rotation. But he was involved in war work from 1942 to 1946 and it was only in the latter year that he became seriously committed to protein research. In England, Max Perutz had already published his first results on hemoglobin crystals (Bernal et al., 1938) but he was interned in the spring of 1940 after the Germans invaded Belgium and Holland and eventually was held in a camp in Canada along with many others who had originally come to Britain as refugees from the Nazis (Judson, 1979; pp. 541–542). The absurdity of this was quickly recognized and he was back in Cambridge in January 1941. A year later he joined Bernal on the HABBAKUK project.

One place where vigorous protein research continued—in fact, the pace accelerated—was Harvard University Medical School's department of physical chemistry. This group managed to make protein chemistry part of the war effort, being recruited by the military already in 1940, before the U.S. became actually involved, to study all aspects of blood transfusion, e.g., purification and storage of serum albumin, which could then be dissolved in water as a plasma substitute. One result of the work of this group was the already-mentioned protein treatise by E.J. Cohn and J.T. Edsall, published during the war in 1943. It was based on more than 20 years work in the Harvard department and included a vast amount of theoretical discussion, with outstanding physical chemists as contributing authors. But the question of the underlying stability of globular proteins was not one of the themes. There was no mention of hydrophobic forces and even intramolecular hydrogen bonds (Mirsky & Pauling, 1936) are mentioned only in one brief footnote.

Entropy and enthalpy

We come next to a most unlikely contributor to this story, Henry Frank, a Christian missionary in China, who had a Ph.D. in physical chemistry and taught chemistry at Lignan University. He was interned by the Japanese after Pearl Harbor but was repatriated in 1942 as part of an exchange and was eagerly hired as an instructor at the University of California to do some of the teaching left uncovered by permanent faculty who had gone to do war work. He was, of course, also free to do research and returned to a subject that had long been on his mind, the derivation by statistical mechanics of equations for thermodynamic data of liquids. The idea was to use a popular (though approximate) approach, in which entropy values are interpreted in terms of "free volume," i.e., the volume over and above the volume occupied by atoms themselves. This was an attractive concept for Frank because it permits (in his own words) a "pictorial interpretation" (Frank, 1983). The first paper dealt with monatomic crystals, the second with pure liquids, the third, with computing assistant Marjorie Evans as co-author, focused on liquid mixtures (Frank & Evans, 1945). Here aqueous solutions immediately stand out as anomalous, for ionic solutes as well as the nonpolar ones which are the main concern here. For nonpolar solutes, the most striking common feature is a *negative entropy of mixing* and an absence of the positive heat of mixing that should accompany the breaking of H_2O–H_2O hydrogen bonds. The result means that hydrogen bonds do not remain broken, but become rearranged in a more restricted, more ordered pattern. Berkeley's most famous chemist, G.N. Lewis, with whom Frank discussed the work, proposed the name "iceberg" for these ordered regions, with no implication of any detailed resemblance to the structure of an ice crystal, but simply as an illustration of the kind of organization that the thermodynamic data required.

Henry Frank knew nothing about protein chemistry, nor probably about orientation of amphiphilic solutes at surfaces—he does not even use the word "hydrophobic" when referring to "rare gases and other nonpolar molecules." Yet his work had a catalytic effect. It was, in a sense, a gloss on the definition of the word "hydrophobic," an illuminating clarification.

The anomalous thermodynamic behavior of systems like water-alcohol mixtures that Frank and Evans were seeking to interpret had actually been already known for some time (e.g., Butler, 1937), and an anecdote told by Kauzmann (1993) is of interest in that connection. He recalls a sabbatical stay with Kai Linderstrøm-Lang in Copenhagen in 1957, after his first paper advocating hy-

drophobicity for protein chemists but before the second, more widely read one. Linderstrøm-Lang appreciated the logic of the hydrophobic concept but thought that, if it were real, it was strange that the heat of mixing of water and plain ethyl alcohol is negative, contrary to what would be expected if the ethyl groups break hydrogen bonds in the water continuum. "The light finally went on," says Kauzmann and he went back to re-read the Frank and Evans paper, which he, a physical chemist at the time of publication, not yet fully committed to protein research, would most likely have noticed in routine examination of current journals. (On the other hand it is unlikely that the *Journal of Chemical Physics*, the most theoretical of all journals related to chemistry, would normally have been read by anyone concentrating on protein research.)

To cut a long story short, a major difference between the two papers by Kauzmann (1954, 1959) is that the second one includes a detailed discussion of the entropy factor, with emphasis on Frank and Evans and the "icebergs" they envisaged. Somehow this "pictorial interpretation," crude as it was, captured the imagination of protein chemists—it provided evidence for the disproportionate strength of hydrogen bonds in water and engendered confidence in the hydrophobic concept. Max Perutz, for example, who was a research student of John Bernal and could therefore have learned about hydrophobicity from him, has the following recollection (M. Perutz, pers. comm.):

> "I cannot remember what Bernal said about hydrophobic groups in proteins but I do remember being very impressed by Kauzmann's review because he gave a reason why hydrophobic groups would be buried and drew my attention to the crucial paper by Frank and Evans, which otherwise I would never have seen."[4]

General acceptance

In the 1940s and early 1950s, the dominant theory for the creation of collapsed protein molecules was the internal hydrogen bond theory. The papers by Pauling and coworkers on the α-helix and β-sheet (Pauling & Corey, 1951; Pauling et al., 1951)—created by intramolecular hydrogen bonds—were the definitive word on the structure of the polypeptide chain backbone. They could not by themselves explain the compactness of globular proteins but it was natural to think that other hydrogen bonds (primarily between polar side chains) would complete the job. But claims for the existence of such links were purely speculative and experimental support for them that was sometimes claimed was usually specious. The interpeptide hydrogen bonds of the α-helix and β-sheet by contrast were backed by 20 years of intense thought and structural studies using model compounds: Hager's biography of Pauling gives a good account of his preoccupation with the subject (Hager, 1995)

Examination of reviews and published symposia during this period indicate little interest in the hydrophobic theory for protein collapse until Kauzmann brought it into the protein mainstream. A scholarly review by Neurath et al. (1944) succinctly states the conventional view: "Native configurations may be considered held together by *attractive* forces of a relatively weak character. The polar groups on the side chains presumably participate in these loose bonds and it is precisely they which are exposed when bonds are loosened by denaturation. The nature of the loose bonds is at present unknown." Isolated statements hint at knowledge of the possibility of a hydrophobic force (e.g., Bull, 1941; Palmer, 1944), but never do so unambiguously or with the implication that a powerful general principle may be involved. Thus Bull (1941), in the body of an exceptionally probing review, points out that "proteins contain a large number of non-polar groups and it might be that upon denaturation these hydrophobic groups are exposed." But his summing-up at the end of the paper gives only salt bridges and hydrogen bonds as relevant energetic factors. A later review by Waugh (1954) on protein-protein association is an exception in that it does embody most of the basic hydrophobic concepts, but they are buried in a welter of distracting and often irrelevant detail and do not seem to have been intended as a vehicle for theoretical insight.

Mostly there is no mention of hydrophobicity at all—as in an otherwise expert review on denaturation by Anson (1945), for example. Likewise a Cold Spring Harbor Symposium on proteins held in 1950, in contrast to the 1938 symposium cited earlier, makes no mention of hydrophobicity, even though there were papers presented which, when viewed retrospectively, were crying out for interpretation in those terms. More generally, one can, for example, cite Pauling, who had many opportunities to express new ideas that might have been in his head, e.g., in relation to his popular theory of antibody specificity (Pauling, 1945) or in general lectures he was frequently invited to give (Pauling, 1948). He often stressed the concept of complementarity (surfaces fitting together) but he always thought of the attraction between the surfaces as due to direct affinity between atoms in contact and there is nowhere any hint of anything else. Linderstrøm-Lang, too, notwithstanding the illumination he later provided for Kauzmann (see above), did not appreciate the hydrophobic principle in 1951. His Lane Medical Lectures (Linderstrøm-Lang, 1952) at Stanford University, for example, included a listing of the forces he saw as being responsible for internal cohesion of proteins but the hydrophobic force was not included as a possibility. In another paper, Jacobsen and Linderstrøm-Lang (1949) give experimental evidence excluding salt links as a possibility and suggest that "we must therefore look elsewhere for a general explanation of the stability of the molecules of protein in solution." They appear to think that their stand was original and make no reference to Bernal (1939b), who, in fact, also explicitly dismissed salt links but in addition gave the looked-for explanation in terms of the hydrophobic factor.

Even Bernal himself seems to have forgotten about his prewar enthusiasm for the hydrophobic principle. As late as 1958 he gave the introductory lecture at a general discussion of the Faraday Society (Bernal, 1958). In the course of it he states that "It is hardly worth recalling here the kinds of forces we shall have to deal with," but then goes ahead and recalls them anyway: in order of strength they are (1) covalent, (2) ionic, and (3) hydrogen bonds, where C=O···H—N bonds are emphasized. He refers the importance of hydrogen bonds for liquid water (as he should, being one of the original exponents), but there is no mention of his earlier lucid explanation of why this should be a vital factor in protein folding.

[4]Looking back on my own activities at the time, I became an enthusiast for hydrophobic bonds after Kauzmann's first paper, with no apparent need for an interpretation in terms of water structure and not even an awareness of the entropy/enthalpy problem (Tanford, 1957). The probable reason is that our laboratory work at the time had yielded gross anomalies for the acid/base titration of tyrosyl side chains of ribonuclease (Tanford et al., 1955), for which burial in a hypothetical oily molecular interior, inaccessible to water, provided a tailor-made explanation, whereas interpretation in terms of hydrogen bonds between amino acid side chains was hugely implausible.

No one at all, in any reviews or discussions of protein stability or folding before 1959, claims acquaintance with the work of Frank and Evans.

After 1959, however, the hydrophobic theory was universally and almost instantly accepted—tyrosyl-carboxylate hydrogen bonds and the like vanished from the scene after that. The last to hold out was Harold Scheraga, professor at Cornell University, the most prolific enthusiast for hydrogen bonds between side chains in the preceding years. He published a hypothetical three-dimensional structure for ribonuclease as late as 1960 (based on the known primary sequence), in which he had the protein held together by internal hydrogen bonds between polar groups and most of the hydrophobic groups were left dangling out at the surface! (Scheraga, 1960).

Strangely—specters from the past—revisionists have recently appeared, questioning not only the "hydrophobic bond" idea as applied to proteins, but even the intrinsic concept of hydrophobicity. The most extreme example (Privalov & Gill, 1989) comes from a Russian-American collaboration, Peter Privalov from the Institute of Protein Research in Moscow and Stanley Gill from the University of Colorado. They say that they want to "bring us back to the twenties" when the antipathy between nonpolar solutes and water was explained by "like to like" attraction, for which they cite the early theory of soap micelle formation of McBain and Salmon (1920) that was later made obsolete by the work of Hartley (1936), as mentioned earlier in this paper. Privalov and Gill support their reappraisal by asserting that "cyclicity is a general principle of evolution of science." As far as I am able to judge, there is as little merit to their specific arguments about proteins as to their claimed general principle of historical cyclicity. Kauzmann (1993, p. 691) gives some other examples of revisionism and comes to the same conclusion.

Another recent development (Lee, 1985) does not question the concept of the hydrophobic interaction as defined in this paper, but does question the "iceberg" model of Frank and Evans as a theoretical explanation for the negative entropy associated with it. This conclusion is based on dissection of the interaction into several stages, with the gaseous state as reference state and computer simulation by means of scaled particle theory as a critical element for examining what goes on in the liquid. The subject is still a matter for debate and in any case does not affect the basic concept of hydrophobicity as a factor in protein folding.

Concluding comment

It is fair to say that the historical strands outlined here have a significance beyond the narrow scope implied by the title of the paper. Though diverse factors are involved in determining the precise specificity of molecular interactions in biology, the hydrophobic force is the *energetically* dominant force for containment, adhesion, etc., in all life processes. This means that the *entire* nature of life as we know it is a slave to the hydrogen-bonded structure of liquid water. This now commonplace conclusion was not generally understood by biochemists until after 1960 and it was the role of the hydrophobic "bond" in the determination of protein structure that was the watershed in comprehension for all, for the simple reason that academic biochemistry departments were less fragmented then than now. There were fewer formal divisions between geneticists, enzymologists, physical biochemists, etc., and weekly departmental seminar lectures were the rule and were attended by everyone, so that the excitement and potential of new advances was shared.

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