

## Linus Pauling and sickle cell disease<sup>☆</sup>

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### Abstract

The 1949 paper by Linus Pauling et al. [Science 110 (1949) 543–548] describing the discovery of sickle cell anemia as the first molecular disease had a major impact on biology and medicine. Inspired by the scholarly works of John Edsall on the history of hemoglobin research, I present a brief retrospective analysis of Pauling's paper. This is followed by some personal recollections of Edsall and Pauling.

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### 1. Introduction

It is a pleasure to contribute this article in honor of John Edsall, one of the most respected researchers, teachers, and scholars of 20th century science. Edsall is widely known for introducing rigorous thermodynamic, spectroscopic and hydrodynamic methods to the study of proteins [1,2]. As a pioneer of biophysical chemistry, he must have been pleased that this subject has grown to become one of the most active fields of modern research. Less well known, perhaps, is that Edsall was also an exceptional scientific historian. His long-standing interest in hemoglobin led him to examine the literature in detail and write a series of fascinating historical accounts [3–5]. In these articles Edsall did more than simply chronicle the sequence of

discoveries. His deep understanding of the subject matter resulted in a perspective that influenced current research on hemoglobin. The style of Edsall's articles is extremely appealing, with intriguing anecdotes and interesting descriptions of what a particular investigator knew, did not know, and, frequently, what he or she should have known. Because hemoglobin has been at the center of the history of biochemistry, these articles [3–5] are highly recommended reading for scientists from any field of chemistry and biology.

Edsall covered the period beginning with the measurements by Christian Bohr in 1904, which showed that oxygen binding is cooperative, to research up to 1970. In his 1980 article [4] he only briefly mentioned the question of whether cooperative binding arises primarily by a sequential mechanism introduced by Pauling [6] and later elaborated by Daniel Koshland, George Nemethy and David Filmer [7], in which binding of oxygen

<sup>☆</sup> This paper is dedicated to John T. Edsall.

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to one heme changes the affinity of its neighboring hemes by a direct interaction, or by the indirect allosteric mechanism of Jacques Monod, Jeffries Wyman and Jean-Pierre Changeux (MWC) [8], in which hemoglobin exists in an equilibrium between high and low affinity conformations that is biased toward the high affinity conformation as successive molecules of oxygen bind. Edsall explained to me shortly after his article appeared that even though the experimental evidence by 1980 favored the MWC mechanism, in his role as an historian it was ‘premature’ to reach a definite conclusion. This was a motivating factor for my writing an historical perspective in the Edsall style, which emphasized research subsequent to 1970 [10]. This perspective describes how the 30-year controversy on the cooperative mechanism that followed Max Perutz’s 1970 landmark paper [9] on the structural mechanism has been settled [10,11], confirming Edsall’s suspicion that the MWC mechanism would turn out to be the correct one. A retrospective view has also recently been published by Robert Shulman [12], who with John Hopfield and Seiji Ogawa carried out an incisive analysis of existing data and performed key experiments in the early 1970s supporting the MWC model [13].

In this second attempt to imitate Edsall, the focus will be primarily on a single publication. It is the famous 1949 paper by Pauling, Harvey Itano, Jonathan Singer and Ibert Wells on the discovery of the first molecular disease, sickle cell anemia [14]. My interest in understanding the history of Pauling’s discovery stems from my own work with James Hofrichter on sickle cell disease. One stylistic difference with Edsall’s papers [3–5] is that there will be more speculation about what Pauling knew, did not know, and possibly should have known. This is obviously risky, since Pauling was an intellectual giant. So corrections to my speculations may be in order.

There are three aspects to Pauling’s 1949 paper which will be discussed. Each one is related to one of the three sections of the ‘Discussion’ in the paper. The first is the little known retraction by Pauling five months later of his interpretation that the charged amino acid content in sickle hemoglobin is different from that of normal hemoglobin

[15]. The second is his explanation of the relation between sickling and oxygen pressure, and the third is his observation of unequal amounts of normal and sickle hemoglobin in sickle cell trait, which he could not explain.

## **2. What Pauling knew about sickle cell disease in 1949**

Pauling first heard about sickle cell disease in 1945. According to his own account he met the noted hematologist William Castle while serving on a government committee planning the direction of post-war research on science and medicine [16]. Castle told Pauling at dinner one evening about sickle cell disease and about experiments which showed the relation between sickling and oxygenation. In Pauling’s words [16]:

‘I had only a mild interest in what he (Castle) was saying, because at the time cells seemed to me to be far too complex to permit me to make any sort of attack on them. However, when Dr Castle said that red cells in the blood of a patient with this disease are sickled in the venous blood but not in the arterial blood, the idea occurred to me that sickle-cell anemia might be a disease of the hemoglobin molecule.’

Castle also told Pauling about the work of a medical student, Irving Sherman, working in the laboratory of Maxwell Wintrobe at Johns Hopkins Hospital, another famous hematologist of that period [16,17]. Sherman carried out an impressive series of experiments on the effect of oxygen pressure on sickle cells. He essentially proved that deoxygenation induces sickling and that reoxygenation restores the red cell to its normal shape. The two telling sentences in Sherman’s paper that surely piqued Pauling’s interest are [17]:

‘The vacuum technique adds further support to their (previous researchers) hypothesis as to the basic role of oxygen, but the actual mechanism of the sickling process remains unknown. In this connection we wish to report without interpretation the observation that under the polarizing microscope characteristic sickle cells exhibit a definite birefringence which disappears after aeration of the cells and the consequent return to the normal discoid form.’

Pauling had been working on hemoglobin since the early 1930s. He had used his magnetic suscep-

tibility measurements and his newly developed quantum mechanical theory of chemical bonding to describe the electronic structure of the iron–oxygen bond [18]. Furthermore, in an extraordinary paper in 1935 he predicted, albeit incorrectly, that the hemes would be in a square-symmetric arrangement on the molecular surface, in sufficient proximity to directly interact and alter oxygen affinity by changing the electronic resonance of the porphyrin ring. Finally, Pauling's experimental research up to that point was mostly concerned with X-ray crystallography. The combination of hemoglobin oxygen release and binding causing sickling and unsickling, and the observation of crystalline order in red cells, must have made the sickle cell problem appear irresistible to him.

In addition to the work of Sherman, Pauling was aware of the prevailing idea that sickled cells can block the microcirculation, as well as cause a hemolytic anemia. Blockage reduces oxygen delivery to the tissues and can result in widespread organ damage and sporadic episodes of severe pain, called 'crises'. Castle presumably also told him about support for this idea from his own studies on increased viscosity of blood and decreased filterability of cells, which indicated that sickled cells are rigid [19]. Other sickle cell papers referenced by Pauling et al. [14] include two on sickle cell trait [20,21], and one showing that red cells without hemoglobin do not sickle [22].

### 3. 'Discussion'

The discussion section of this classic paper addresses three separate aspects of the significance of the two major experimental results—the finding of a difference in electrophoretic mobilities of normal and sickle hemoglobin and the finding of comparable but not equal amounts of sickle and normal hemoglobin in individuals with sickle trait.

#### 3.1. 'On the Nature of the Difference between Sickle Cell Anemia Hemoglobin and Normal Hemoglobin'

This first section of Pauling's three-part discussion estimated the difference in the net charge between normal and sickle hemoglobin from the beautiful and carefully controlled electrophoretic

and acid–base titration data obtained by Itano and Singer. Pauling concluded that there is a '...difference in the number or kind of ionizable groups in the two hemoglobins...; sickle cell anemia hemoglobin therefore has 2–4 more net positive charges than normal hemoglobin.' A value of three more net charges was arrived at from the observation of a difference of 0.23 pH units in the isoelectric point, and a change in 13 charges per pH unit from titration curves. Pauling chose 2–4 charges 'with consideration of our experimental error.'

A retraction of the conclusion concerning a change in ionizable amino acids followed almost immediately. It appeared in the following published abstract [15], reproduced here in its entirety, from the April 1950 meeting of the National Academy of Sciences in Washington, DC, 5 months after the appearance of the 1949 paper:

#### 'Sickle Cell Anemia Hemoglobin

Linus Pauling, Harvey A. Itano, Ibert C. Wells, Walter A. Schroeder, Lois M. Kay, S. J. Singer, and R. B. Corey, *California Institute of Technology*

Amino acid analyses of hydrolyzates of normal human adult hemoglobin and sickle cell anemia hemoglobin have shown that there is no difference in the number of residues of the acidic and basic amino acids in these molecules. There may be small differences in the number of residues of leucine, serine, valine, and threonine. Preparations of hemin chloride and the dimethyl ester of the heme porphyrin from the two hemoglobins have been found to be identical. The globin preparation resulting from acid acetone treatment of the two hemoglobins give the same electrophoretic patterns. The difference of 0.23 pH units in the isoelectric point of the hemoglobins cannot be attributed to differences in the number of acidic or basic amino acid residues, but is presumably the result of a change in the ionization constants of acidic and basic groups resulting from differences in folding of the polypeptide chain.

The amount of sickle cell anemia hemoglobin in the erythrocytes of 25 individuals having sickle cell trait has been determined, and values between 25 and 44 percent have been observed.'

The failure to find a change in charged amino acids must have been difficult for Pauling to accept. He had presented a different and much more appealing interpretation of the results on the intact hemoglobin molecule at the National Academy meeting 1 year earlier, adding to his possible embarrassment. Pauling was obviously stumped

and had to use his fertile imagination to preserve the idea that sickle cell anemia was a molecular disease caused by a change in amino acids. His explanation of a different fold in the mutated protein changing the pK's of ionizable groups might have seemed far-fetched to his colleagues. Notice also that Pauling's coworkers performed additional experiments apparently fearing that the difference between normal and sickle hemoglobin may be in the relatively uninteresting hemes. (Pauling reported later that '...there is a difference in electrical charge on the globins approximately the same as that on the two kinds of hemoglobin.' [23])

Interestingly, when the work on the amino acid composition was completed and the full paper published a short time later with the same negative result [24], Pauling's name does not appear among the coauthors, although he and Robert Corey did receive gracious acknowledgements. Perhaps Pauling did not want to abandon his wonderful idea.

Six years later Vernon Ingram invented peptide 'fingerprinting', in which the composition of small peptides derived from the partially digested protein could be much more easily analyzed. In 1956 he described his discovery of a peptide fragment with a different net charge in sickle and normal hemoglobins [25] and a year later reported that in this fragment a negatively charged glutamate in each of the two beta chains of the hemoglobin molecule was replaced by a neutral valine, neatly explaining the net increase of two positive charges predicted by Pauling [26]. Ingram recently told me that he had no communication with Pauling concerning his experiments, in part because he was working in Cambridge under Perutz (preparing heavy metal derivatives when he was not working on his own project of determining the chemical difference between normal and sickle hemoglobin), in the same group with John Kendrew and Francis Crick, who regarded Pauling as a competitor and a 'genius.'

### 3.2. 'On the Nature of the Sickling Process'

In this section of the Discussion, Pauling indicated that the experiments essentially prove that the aggregation of an abnormal hemoglobin is

responsible for the sickling process and the disease [14]: '...(aggregation) supplies a direct link between the existence of 'defective' molecules and the pathological consequences of sickle cell disease.' His aggregation mechanism derived from his previous ideas on complementarity in antigen–antibody reactions [27], leading him to suggest '...that there is a surface region on the globin of the sickle cell anemia molecule which is absent in the normal molecule and which has a configuration complementary to a different region of the surface of the hemoglobin molecule.' With typical Pauling inventiveness, he then invoked a coupling of the conformation of either the donor or acceptor site to the state of oxygenation of the hemoglobin molecule. Pauling assumed that the surface region was close to the heme, indicating that he did not think that conformational changes could be propagated to very distant sites. This was also an assumption in his rejection of the (correct) tetrahedral arrangement of the hemes in his ingenious 1935 analysis of the cooperative oxygen binding curve [6]. Nevertheless, Pauling had essentially the right idea about the effect of oxygen binding. He, of course, did not know that hemoglobin exists in two quaternary structures, which was discovered in the early 1960s by Perutz [9] and is the key to explaining the effect of oxygen on polymerization and sickling.

We now know that the results on polymerization and oxygen binding can be quantitatively explained with the simple idea that the T quaternary structure, favored by deoxygenation, polymerizes, while the R quaternary structure, favored by oxygenation, does not [28]. An analysis of the X-ray structure of deoxyhemoglobin S, moreover, shows that the R quaternary structure simply does not fit into the strands that make up the deoxyhemoglobin S polymer [29].

### 3.3. 'On the Genetics of Sickle Cell Disease'

One of the major conclusions from the finding of nearly equal amounts of normal and sickle hemoglobin was that the gene responsible for sickling is in the heterozygous condition in the benign sickle trait and in the homozygous condition in sickle cell anemia. Neel [21] had already

reached this conclusion from his observation that sickled cells were always found in the blood of normal (sickle trait) parents of a sickle cell anemia patient. Nevertheless, Pauling claimed [14]: ‘Our results had caused us to draw this inference before Neel’s paper was published.’

The problem facing Pauling was to explain the 40:60 ratio of sickle to normal hemoglobin rather than the 50:50 ratio expected in the simple heterozygous case. Again he used his creative mind to come up with schemes, but there was so little known about the complex process of protein synthesis that his explanation was necessarily vague, having to do with competition by the sickle and normal genes for substrates involved in protein synthesis. In the last sentence of the 1950 abstract, the ratio is reported to be as small as 25:75, with not even a comment. It is clear from Pauling’s later papers on proteins that he remained perplexed by these results [30].

We now know that there is no difference in the rate of sickle and normal beta chain synthesis [31], and that the deviation from a 50:50 ratio can be understood in terms of assembly of the hemoglobin tetramer [32]. The explanation came with the observation by the biochemist/hematologist H. Franklin Bunn. From studies of many mutant hemoglobins, Bunn showed that the assembly of the positively charged alpha chains is less efficient with beta chains that are more positively charged, as in sickle hemoglobin with its neutral valine at the beta 6 position compared to normal hemoglobin with a negatively charged glutamate at beta 6 [32]. He further showed that this difference is exacerbated when fewer alpha chains are synthesized because one or more genes coding for the alpha chain are missing (alpha thalassemia), thereby explaining the 25:75 ratio. Pauling could not have guessed at this, since alpha thalassemia was unknown in 1949 and it had not yet even been established that hemoglobin consists of alpha and beta chains.

#### 4. Closing remarks

The 1949 paper is filled with new insights and brilliant guesses, and was an extremely influential scientific work. Pauling was not, however, able to

completely solve the problem at the conceptual level. Most importantly he had no suggestions on how to treat the disease. Perhaps he realized that blocking intermolecular contacts was not a viable approach because of the massive amount of a drug that would be required. In the early 1960s, when I heard him lecture on sickle cell disease at the University of Pennsylvania School of Medicine, he spoke only of genetic counseling.

Strategies for therapy required understanding the kinetics of sickling, which apparently did not concern Pauling. The medical implications of the kinetics came with the discovery of a delay time prior to the appearance of polymer that is enormously sensitive to solution conditions, depending inversely on the 30th power of the initial hemoglobin concentration [33,34]—the highest concentration dependence ever found for any kinetic process in solution. The delay time makes sickle cell disease survivable because the vast majority of red cells, which would otherwise be sickled at tissue oxygen pressures, escape the narrow vessels of the microcirculation before polymerization has begun [35]. The kinetic studies led to a coherent description of the pathophysiology in which the probability of microvascular occlusion, and therefore clinical severity, is determined by the relation between the delay time and the transit time of red cells through the microcirculation [33,36]. This dynamical description of the pathophysiology immediately suggested that diluting sickle hemoglobin or preventing it from concentrating should be an effective treatment of the disease [33,36]. These ideas explain, moreover, why replacement of only approximately 20% of the hemoglobin of a cell with the non-polymerizing fetal form has a therapeutic effect [37,38], as shown in a large-scale clinical trial with hydroxyurea, a drug that stimulates fetal hemoglobin synthesis by an as yet unknown mechanism. Diluting the sickle hemoglobin with fetal hemoglobin results in a more than 1000-fold increase in the delay time, allowing the cells to escape the microcirculation prior to sickling [37,38]. A completely different and promising approach to therapy based on these kinetic ideas is to prevent sickle hemoglobin from concentrating via the cellular dehydration that results from the damage of sickling. The physiologist/hematologist

Carlo Brugnara has recently developed two classes of drugs that prevent potassium and water loss from sickle cells, and has obtained promising results in clinical trials [39].

It has now been over 50 years since Pauling's discovery of the molecular cause of sickle cell disease [14]. Only now, however, are we on the threshold of a truly effective treatment. This long search for a cure of sickle cell disease contains many lessons for current medical research with its explosion in the discovery of disease-causing genes.

### 5. Personal recollections of Edsall and Pauling

My first connection to Edsall came while I was a medical student at the University of Pennsylvania in the early 1960s interested in biophysical chemistry, an interest generated by summer research projects on calorimetry and potentiometry supervised by Philip George and his post-doctoral fellow Abel Schejter. Edsall and his close colleague and life-long friend, Jeffries Wyman, wrote an excellent textbook of biophysical chemistry [2] which I read with great interest. I had no personal contact with Edsall until he and Wyman came to the NIH in 1970 as Fogarty Scholars-in-Residence. During their stay they organized an historic meeting on allosteric proteins at Stone House on the NIH campus. My research interest at the time was an extension of my Ph.D. thesis research with Robin Hochstrasser at Penn, and concerned the interpretation of the electronic spectra of hemoglobin in terms of molecular orbital and crystal field theories, a subject of little interest to the participants at the meeting. However, I was keen to learn whether I could use my knowledge of hemoglobin spectroscopy to investigate the mechanism of cooperative oxygen binding, one of the most intensely studied and controversial subjects in biochemistry at the time. Edsall kindly arranged for me to attend this rather exclusive meeting, for which I am indebted to him, since the meeting sparked my long-standing interest in the hemoglobin mechanism.

It was not until 1974 that I met Edsall again, when I was invited to present a seminar on sickle cell disease in the Biological Laboratories at Har-

vard. My contacts with him did not become frequent, however until the spring semester of 1976, when I was a visiting professor in biochemistry, sharing the teaching of a biophysical chemistry course with Stephen Harrison. My office was located in the Gibbs Memorial Laboratory (Wolcott, not J. Willard). I was also named the first (and last) 'Gibbs Visiting Professor' by the self-appointed president of the Gibbs Society, Gibbs historian, and graduate student working with Steve to solve the X-ray structure of tomato bushy stunt virus, the remarkable Clarence Schutt, known simply as Schutt. On Gibbs Day in 1976 Edsall, always the austere gentlemen, showed his sense of humor by leading a march of graduate students, post-doctoral fellows, and faculty with Schutt, who was clad in a strikingly white Victorian-style suit, from the steps of Gibbs to a rather tacky fish restaurant in southeast Cambridge. After lunch, to the delight of all in attendance, Edsall made several quite amusing remarks about Gibbs' work, in response to Schutt's selected readings from Gibbs' least interesting publications.

Since that time my visits to Cambridge invariably included a visit with Edsall. Our common interest was the physical chemistry of hemoglobin. Edsall, with his encyclopedic knowledge of protein physical chemistry, was an invaluable source of information and insights in the field. Biophysical scientists of my generation constantly sought his opinion on their new experimental findings or theoretical ideas. Each of my encounters with him in Cambridge or in Bethesda was marked by lively and intense discussions on the latest advances and controversies in hemoglobin research. Because of his impeccable ethics in all matters related to science, it was also always extremely interesting to discuss the changing ethics of modern research.

One particularly memorable meeting was on the occasion of a visit to the NIH in April of 1980 by Edsall and Pauling, organized by my colleague, Gary Felsenfeld, an undergraduate research student of Edsall at Harvard, and a graduate student of Pauling at Cal Tech. Following Edsall's lecture Gary arranged to have me present a seminar in the library of our small research building (Building 2) to Edsall and Pauling on the research that I had done on sickle cell disease in collaboration with

James Hofrichter and, in the initial experiments, with Philip Ross, from the neighboring Laboratory of Molecular Biology in Building 2. Edsall already knew about our work from our papers and many previous discussions, but Pauling apparently did not. I began my presentation by showing a picture of the 14-stranded helical fiber determined by Stuart Edelstein and coworkers using reconstruction analysis of electron micrographs [40]. I then went on to describe our work, including the most recent experimental results. In his characteristically reserved and scholarly manner Edsall asked detailed and penetrating questions about the thermodynamics and kinetics of the polymerization process. Like Edsall, I expected Pauling to be excited by our discovery of the 30th power concentration dependence of the delay time and its medical implications. Not so. In retrospect I should not have been surprised and disappointed. Pauling's scientific interests were chemical bonding, molecular structure, and a wide range of biological and medical subjects. He apparently did not spend much time in his research career thinking about kinetics. Nevertheless, he seemed impressed and accepted our explanation of a double nucleation mechanism to account for the high time and concentration dependence [41] and appreciated its relevance to the pathophysiology of sickle cell disease. But at almost 80 years, Pauling was totally immersed in his research on vitamin C as a way of prolonging life, and his only question about the kinetics was whether sickle cell disease could be treated by increasing the delay time with vitamin C. Fortunately, I had anticipated such a question and was able to give him a detailed answer, since the week prior to Pauling's visit Jim and I had performed a series of kinetic measurements which showed that vitamin C did not decrease the rate sufficiently to be therapeutic.

My conversation with Pauling at a cocktail reception was much more rewarding. Our discussion ranged from the electronic structure of the iron–oxygen bond of hemoglobin [42–44] and the birefringence of sickled cells [14,45] to the evolution of its amino acid sequence [30,46]. The day ended with a dinner at an Italian restaurant in Bethesda. One of the memorable comments from the dinner was made by Pauling's wife Ava, who

chided her husband about the race with Watson and Crick to solve the three-dimensional structure of DNA by saying: 'Linus, I told you that you should have worked harder on that problem.'

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