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Dielectric behavior and atomic structure of serum albumin

J.L. Oncley*

Biophysics Research Division, University, of Michigan, 930 N. University Avenue, Ann Arbor, MI 48109-1055, USA

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Abstract

After 1946, serum albumin was available for studies. Its residue sequence and internal disulfide bonding was developed by 1976. We began to make dielectric dispersion studies and apply Perrin's equations for rotational relaxation times around the two axes of revolution in 1938. These data indicated that albumin should have an elongated shape. In 1992 atomic structure data indicated the molecule was heart-shaped. A similar 1998 study of albumin complexed with fatty acid showed that the molecule was substantially rearranged. We found that the dielectric constant of albumin solutions was sensitive to fatty acid content, making this property an attractive probe in stop–flow kinetic studies. Such studies show that the fatty acid reaction is a two-step process. The fatty acid first binds to exterior sites in a diffusion-limited second order reaction complete in 1 ms. Then a first order rearrangement reaction with ~400 ms half-life follows. Thus the highly specialized serum albumin sequence of amino acid residues determines not only the structure of the unligated molecule, but also the distinctive structures of the numerous multiligated molecules.

Keywords: Serum albumin; Fatty acid complex; Stop–flow kinetics; Dielectric properties; Rotary Brownian motion; Perrin relaxation times

1. My first involvement in the Harvard laboratory

My interactions with John T. Edsall were strongest in the period from ca. 1937 or so to 1953 when we were both members of the Cohn group. It was in the Harvard Medical School, Department of Physical Chemistry (1920–1950); Harvard, University Laboratory of Physical Chemistry (1950–1953); and Harvard Medical School, Department of Biophysical Chemistry (1950–1956). All of these organizations were dominated by Edwin J. Cohn, and I will refer to all of them in this manuscript as COHN LAB.

According to Professor Edsall's article *A Historical sketch of the Department of Physical Chemistry, Harvard Medical School; 1920–1950* in the American Scientist in 1950,

> ''...Jeffries Wyman, Jr. ...came to work on proteins in 1927 and later returned to the department of biology at Harvard. While he was in this laboratory, arrangements were made for him to study the unusual dielectric properties of

*Present Address to which correspondence should be addressed: The Harwich House, #8, 26 Pleasant Lake Avenue, Harwich, MA 02645-2446, USA..

E-mail address: jl.oncley@verizon.net (J.L. Oncley).

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amino acid and peptide solutions, with our advice and collaboration. In the laboratory of Professor G.W. Pierce of the physics department at Harvard University, Wyman developed an apparatus beautifully adapted for the study of these systems''.

I still remember seeing two adjoining articles in *Physical Reviews* while at the University of Wisconsin in 1930, the first by F.H. Drake, G.W. Pierce and M.T. Dow which gave the dielectric constant of water at 25 °C to be 78.57, and the second by Jeffries Wyman, Jr. which gave 78.54 for the same quantity. Both of these values were at least one decimal more accurate than previous determinations. The first value was obtained with a water-containing cell perhaps 4 m or so in length, while the second used a cell more, nearly 4 cm long, suspended in a beaker of water by a small string.

My own introduction to the laboratory was very gradual. The year 1932 marked the peak of the Great Depression, and jobs were very hard to come by. I received a National Research Council Fellowship in Chemistry to work with Prof. F.G. Keyes at the Department of Chemistry, Massachusetts Institute of Technology (MIT) in the academic years 1932–1934, and had attended a few seminars at Cohn's Laboratory, mostly to meet Wyman and make a date to see his laboratory in the Biology Department.

In 1934–1935 I had a two semester appointment as Instructor in Physical Chemistry at the University of Wisconsin taking over the duties of Prof. J.W. Williams, who had been my major professor and was on sabbatical leave after receiving an International Health Board Fellowship from the Rockefeller Foundation to do research in Uppsala, Sweden on the dielectric properties of proteins. I needed a job after he returned, and at his suggestion applied to the Rockefeller Foundation to work with Prof. E.J. Cohn at Harvard Medical School. However, I had an offer of a job as Instructor of Chemistry at MIT before a decision regarding the fellowship was made, which I immediately accepted. When I wrote to Prof. Cohn and the Rockefeller Foundation to withdraw my application for the fellowship Prof. Cohn suggested that he might be able to provide some of the equipment that I needed for the dielectric studies on proteins, and help in the provision of suitable protein solutions for me to pursue this work in my research time at MIT. I accepted this offer and set up apparatus in my office at MIT in the fall and winter, and made preliminary experiments with dilute salt solutions, but when I started to use protein solutions I found it much more convenient to move my apparatus to COHN LAB in a space of approximately 1 m^2 in a laboratory adjacent to Prof. Cohn's office. I held no official Harvard appointment until I was hired to build and operate an ultracentrifuge in February 1939, but Cohn and Edsall have always called 1935 or 1936 the year that I joined the Harvard laboratory.

I include these paragraphs to illustrate how Prof. Cohn operated to get things done in COHN LAB. In the early 1930s Cohn wanted to study the dielectric constants of amino acids and peptides, and Wyman developed a method using very high frequencies for this purpose, working in the Harvard Physics Laboratory. In the late 1930s Cohn wanted to return COHN LAB to protein studies, and I developed the bridge method at the much lower frequencies needed for this purpose working in the MIT Chemistry Department. In both cases Cohn provided funds and help with suitable materials for measurements regardless of departmental and institutional barriers.

I was delighted to be asked to review John Edsall's contributions to science on the occasion of the presentation of the 1966 Passano Award at a dinner in Chicago, and to attend the symposium in honor of his 90th birthday in 1992 at the American Academy of Arts and Sciences in Cambridge.

2. Serum albumin

In the period after World War II serum albumin, usually of either human or bovine origin, was probably more often investigated by physical and biophysical chemists than any other protein. COHN LAB made it easy to obtain small samples of proteins for broad experimental use, and also had many laboratory visitors for 1- or 2-year periods until the death of Professor (later Huggins University Professor after July 1949) Edwin J. Cohn on 1 October 1953.

At COHN LAB, Walter L. Hughes discovered that much of the albumin could be crystallized as

a mercaptalbumin form (with one free –SH group which formed a dimer when treated with the proper amount of mercuric ion, and Jacques Lewin (1947–1951) prepared many crystalline derivatives with various metals. Margaret Hunter joined the COHN LAB in 1948 and continued to study albumin homogeneity in our University of Michigan Biophysics Research Division when we moved there in 1962. Barbara Low, who worked with Dorothy Crowfoot (later Dorothy Hodgkin) on the penicillin structure, started X-ray structure studies on serum albumin at the COHN LAB in 1948, and continued her interest on the atomic structure of albumin when she moved to the Department of Biochemistry at Columbia University College of Physicians and Surgeons. We all hoped for, and expected, an early success after the myoglobin–hemoglobin success just prior to 1960.

In those early days protein crystals usually had 50% or more peptide content, and X-ray atomic structure analysis slowly became more or less commonplace. But without success for serum albumin of any species. Then came a period of investigation in space shuttle crystal growing, new solvents, etc. The first success with albumin came in 1989, when crystals that contained only 22% or so of peptide were obtained from 20% polyethylene glycol solution. They also worked with carefully defatted albumin, and albumin from recombinant RNA synthesis in bacterial systems. To my knowledge, no albumin crystals grown in the space shuttle were more successful than those grown on planet Earth, although the original success by Carter and He in 1989 was carried out in The Space Science Laboratory in Huntsville, Alabama.

3. Atomic structure of human serum albumin

I was very pleased to see an elongated ellipsoid-shaped human serum albumin molecule located diagonally across the unit cell illustrated on the cover of *Science* in 1989. Inside was a paper [1] which announced a very low resolution (approx. 6–7 Å) study of human serum albumin where they had traced a single peptide chain from start to finish. The ratio of the molecular length to width was approximately 4, within the error limits we had predicted from studies of the dielectric dispersion of fatty acid free human serum albumin [2]. My period of pleasure lasted less than 12 months when a retraction of the 1989 *Science* paper appeared [3]. This report stated that they had skipped from one peptide chain to another at an ill-resolved position, after which they followed the adjoining chain of a second molecule. They didn't say that they had been suckered into publishing an incorrect model for serum albumin from very preliminary data because it seemed to agree with an overwhelming amount of data favoring the elongated ellipsoidal model. But that must have been the case in 1989!

In a 1992 study He and Carter [4] achieved a resolution of 2.8 Å and concluded that the human serum albumin molecule had the sequence and disulfide binding assigned by Brown [5], was heart-shaped, and could be approximated to a solid equilateral triangle with sides of approximately 80 Å and average depth of approximately 30 Å.

4. Fatty acid binding

Prior to World War II it was thought that the principal function of serum albumin was to maintain the osmotic pressure of the plasma. One of the early new findings concerning human serum albumin was that albumin molecules were capable of binding a great variety of metabolites and drugs, including fatty acids. It was then found that a whole new source of metabolic activity was carried out in the blood by oxidation of fatty acid. Much of the early work on the importance of this tiny fraction of free fatty acid in the blood, and its transport by albumin was conducted in the laboratories of Vincent P. Dole of the Rockefeller Institute (later Rockefeller University) and Robert S. Goodman of the National Institutes of Health.

In 1998 a crystal structure of human serum albumin complexed with five molecules of myristate was published by Curry et al. [6]. They found the fatty acid molecules were bound in long hydrophobic pockets capped by polar side chains,

many of which are basic. These pockets are distributed asymmetrically throughout the molecule, despite the albumin's symmetrical repeating structure. They also found that a large fraction of the peptide chain around the fatty acid ligands was shifted from the its unligated position.

5. Dielectric constant studies

A homogeneous protein molecule in a suitable solvent will undergo two types of Brownian movement, linear and rotational. For the case of ellipsoidal shaped molecules both of these forms of Brownian motion have been treated by F. Perrin[1] [7,8]. For rotational motion he found that elongated ellipsoids would have two quite different relaxation times whereas flattened ellipsoids would have two nearly equal values.

In 1936–1939, while an Instructor at MIT's Chemistry Department, I was able to modify a newly available commercial radio frequency bridge, with help from Robert F. Field and Donald Sinclair of the General Radio Company, so that it would measure the capacitance of a suitable cell as a function of the frequency in the range from 0.025 to 2.5 MHz and obtain the dielectric constant to an accuracy of approximately 0.1%. The high frequency range was increased to 6.3 MHz in later work and I was greatly aided by the work of my first post-doctoral student Norman Hollies. The first protein that I studied was horse carboxyhemoglobin [9]. It was found to have a single relaxation time as expected for an almost spherical shape, and the proper value for a molecular weight of 66 700 and a hydration of approximately 0.6 g water per gram of protein. It had a dipole moment of 470 Debye units. I now realize that our choice of hemoglobin was a poor one, since much of its dipole moment is canceled because of the symmetry of the $(\alpha\beta)_2$ form of this molecule (not known until 1960, however).

The next molecule that I studied, with the help of John D. Ferry, was horse serum albumin [10] which had two fairly well separated relaxation times, thus indicating an elongated ellipsoidal shape and a dipole moment of approximately 280–510 Debye units, depending on the fraction measured. When I tried to obtain the dipole moment of albumin from different species before giving an American Chemical Society lecture when I received the 1942 Award in Pure Chemistry for this work, I was annoyed by the fact that different preparations of human serum albumin (HSA) gave varying values for their dipole moment. It was my graduate student Howard Dintzis who found, ca. 1951, that this value depended directly upon the long chain fatty acid (FA) combined by each preparation. He established the fact that there was an almost linear relation between dipole moment and FA content after developing a synthetic resin column capable of removing FA and electrolyte from the sample (instead of using electrodialysis as I had previously used). Further work 10 years later by my first biophysics graduate student Walter Scheider confirmed this work, and found that lower values of FA binding by HSA could be obtained by defatting the preparation by action of a biological system. The method used took advantage of the observation that epididymal fat pads of the rat compete strongly, under certain conditions, for even the most tightly bound FA on HSA [11]. The result of all this work was published by Scheider et al. [2]. In this paper values for rotational relaxation times at infinitely low concentration were obtained. This value was somewhat lower than measured at higher concentrations, but did still seem to indicate the elongated ellipsoidal shape. Dipole moments for defated albumin were about 710 D for human, and 460 D for bovine material. Values for oleic acid complexes with human serum albumin were reduced to 600, 510, 470, 435 and 405 D for integral values of oleic

[1] An error in [7] by Perrin, and reprinted by many textbooks including Cohn and Edsall (1934) ''Proteins, Amino Acids, and Peptides''. p. 511 and Alexander and Johnson, (1949) ''Colloid Science'' pp. 385–386 was pointed out by S.H. Koenig in Biopolymers, 14 (1964) 2421–2423. It involves a simple change in sign in two of the equations, and was used with correct sign by myself and others in their calculations before the Koenig note. There are many tables of the values: I provided one of the first in an appendix to ''Proteins, Amino Acids and Peptides'' by Cohn and Edsall in 1934, pp. 644–651. A graphical plot vs. log (a/b) or (b/a) can be found in Cantor and Schimmel, ''Biophysical Chemistry'', Part II, Fig. 10–ll (b), p. 564.

acid between 1 and 5. Bovine serum albumin was measured over a more restricted range from 0 to 1.4 moles of oleic acid to give about 370 D for the 1:1 complex.

The relaxation times and hydrodynamic properties for isoionic bovine serum albumin (BSA or HSA) have been studied by more than half a dozen other laboratories. Typical reports are found in references [12,13]. Depolarization of fluorescence, first studied by Weber [14], does not give two relaxation times, but gives a harmonic mean of the relaxation times. These studies have all given results in agreement with the dielectric studies in as far as they all concluded that application of the Perrin equations [7,8] indicated that the serum albumin molecule seemed to be elongated rather than flattened, although the axial ratio varied somewhat in these reports. Edsall and Foster [15] studied the double refraction of flow of BSA and found further proof of the elongated model.

6. Shape of edestin

At this point, I should introduce an earlier failure to predict the correct shape of the edestin molecule. I had studied some preparations of edestin, a rather large protein extracted from hemp seed that was often prepared in the early 20th century and had been isolated in COHN LAB by Kenneth Bailey in 1939–1940. Edestin was only very slightly soluble in conductivity water, but Bailey had found it fairly soluble in 2 M glycine solution, and I had measured its dielectric properties in that solvent, but had studied its sedimentation velocity properties in salt solution as solvent (as it had been studied before). I found that it had two fairly different relaxation times, and a dielectric dispersion curve shaped like serum γ-pseudoglobulin, and at slightly higher frequencies. While Howard Dintzis had been in COHN LAB we had found a study in the electron microscope that showed that it was spherical. It also crystallized with, I believe, six near neighbor molecules. I wanted to see if the glycine solution might have contained an aggregate of perhaps seven molecules which was responsible for the second relaxation time. Dintzis and I learned then that marijuana was also known as the Indian hemp, and that all hemp seed had to be heat-treated to insure that it would not grow. The only way we could make edestin was to obtain a special permit from the Treasury Department to grow Indian hemp, and we decided that we didn't want to put that much effort into the work.

7. The albumin–ligand reaction

Along with long chain fatty acids, a number of other ligands bind with serum albumin. Because of the very low solubility of the fatty acids in aqueous solution, many of the studies involve more soluble ligands. Extensive studies with thiocyanate ions (SCN^-) indicate that in the pH range from 4.5 to 10 there are 6–10 strong binding sites and approximately 90 sites about 1/70th as strong. Rapier H. McMenamy, whom I inherited as graduate student when Dr Cohn died, had developed a fine method for concentrating non-protein components of plasma by a quantitative dialysis procedure. In a study of recovery of amino acids added to human plasma he found that L-tryptophan in blood plasma was bound by a non-dialyzable plasma component. He identified this component as serum albumin [16] and found that albumin had essentially no affinity for the D-isomer. At that time it was believed to be the only instance in which stereospecificity of such a high degree had been found to occur in binding of compounds to albumin. He also studied the binding of *N*-acetyl-L-tryptophan and L-tryptophan and showed in competitive studies that they were bound at the same site. This was of special interest since *N*-acetyl-DL-tryptophan was used as a stabilizing agent in one of the 25% human serum albumin solutions in clinical use.

8. Stop–flow studies of the reaction

The large variation in dielectric constant of HSA-$(FA)_n$ solutions as a function of FA content, *n*, suggested this property to Scheider as a better probe to study the kinetics of exchange reactions between SA and FA and similar ligands. Conventional optical probes have usually not been useful.

The probe consists of a measurement of the dielectric properties of a reacting mixture, made by recording the response in real time to a single

electrical pulse. The observations are made on rapidly mixed solutions of human serum albumin and ligand, with the use of stop–flow methods. By a Fourier transformation one can go from a description of a system response in the frequency domain, to a description in the time domain. Scheider developed a method that consists of applying a single square pulse of the order of 0.02–0.05 ms duration, across a bridge arrangement which balances out what might be called the base-line capacitance and conductance of the sample. Using custom-designed electronics to permit programming of pulse width, repetition rate, and data-sampling times, the bridge output is amplified and sampled at intervals of 0.2 ms, converted to digital form with 8-bit accuracy, and stored in a memory for retrieval and processing later [17].

In several series of experiments with this method [17–20] he identified two steps in the association of FA with HSA. The two solutions begin to react within 1 ms of reaching the stopped–flow mixer, and the mixture is then passed to the monitoring cell, where rapid dielectric measurements are automatically initiated. Step 1 is complete during the dead time of mixing (approx. 1 ms) and involves the adsorption of FA at a weakly reacting site in the native structure indicated in the equation below by the round brackets (HSA)·FA and involving a reduction of conductance to nearly that of the original SA solution. Step 2 involves an internal rearrangement to a new form of the amino acid residues and FA, indicated by the square brackets [HSA, FA], and this rearrangement follows first-order kinetics and has a half-live of approximately 400 ms. In most experiments this second step is essentially complete in approximately 2 s. To summarize,

$$(\mathrm{HSA}) + \mathrm{FA} = (\mathrm{HSA})\cdot\mathrm{FA} = [\mathrm{HSA,FA}]$$

Step 1 Step 2

The inference from the conductivity data is that the time constant of the initial binding step is less than 1 ms, giving a second-order rate constant $k_1 > 10^6\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$. This suggests a diffusion-limited first step. With ligand and protein already in place, it is to be expected that the subsequent step be of first-order kinetics, as indeed the data suggest.[2]

Further work of Scheider has established the thermodynamic and kinetic specifications of this two-step binding of oleate, linolate and laurate to HSA.

> ''The second step of association is rate limited by a negative entropy of activation, the enthalpy of activation is nearly zero. Thus, minimal bond disruption is required in the association, but a particular configuration of the protein or its solvent environment (or both), having a relatively high degree of order, is required''.
>
> ''Step 2 of association involves a rearrangement of the SA–FA conformation, as shown by the change in the dipole vector. During this step, the ligand gains access to the hydrophobic interior of the protein where the primary binding region is located''.
>
> ''The transient activated state of step 2 is identical, or closely related, to the opening up of the tertiary structure of the serum albumin molecule which is thought to occur in the acid transition''.
>
> ''Dissociation activation requires enthalpy sufficient to break the hydrophobic region as well as a negative entropy

[2] In [17], Fig. 5, a third reaction sequence from 1 to 20 s is shown, and the author states that ''There are several possible explanations of the data from 1 to 20 s which will be elucidated in further experiments''. He left the field before many experiments over this time range were performed, and that figure is the only one in his series of papers using stop–flow methods that were presented in detail, since that time period was not recorded in later work. Unfortunately, the data in Fig. 5 was obtained using *N*-acetyl-DL-tryptophan, rather than FA as ligand. McMenamy and I [16] showed that L-tryptophan was strongly bound to HSA, while in a section titled ''Non-binding of D-tryptophan'' we state ''It was surprising to find that albumin had essentially no affinity for the D-isomer of tryptophan. The small amount of binding observed was of the same order as had been found for the binding of L-tryptophan to its secondary sites. This is believed to be the only instance in which stereospecificity of such a high degree has been found to occur in binding of compounds to albumin''. We also studied the binding of *N*-acetyl-L-tryptophan and L-tryptophan, and showed in competitive studies that they are bound by the same groups. We did not actually experimentally show that *N*-acetyl-D-tryptophan did not bind, but assumed that it did not from the facts already proven. I should add that N-acetyl-DL-tryptophan is used as a stabilizing agent for one of the clinically used HSA solutions, and that we cited two reports that *N*-acetyl-DL-tryptophan had been reported to bind, although details were not published. In Scheider's stop–flow experiment reported in [17], Fig. 5, he reacted solutions of equal molality of human serum albumin and *N*-acetyl-DL-tryptophan, so he would have had an excess albumin and the D-isomer remaining at the 2–30-s period which may have slowly reacted.

change sufficient to bring the protein into the more highly ordered activated state described for step 2 of association above''. (Quotes from Scheider).

This description of the second step in this binding process seems in complete agreement with the X-ray structural studies in the latest work on HSA(myristate)$_5$ [6], where major shifts in the peptide chain are observed as being caused by the addition of the FA myristate.

9. Some additional problems in dielectric studies

Scheider also answered several other basic questions that had been raised regarding dielectric dispersion studies, and which I had been unable to satisfactorily answer. When a dissolved macromolecule is in chemical equilibrium with a free ionic species, the charge configuration, and hence the dipole vector of the macromolecule is fluctuating. Expressions for the static dielectric constant and the relaxation spectrum of such a mixture were derived in terms of the components of the mean moment and the root mean square fluctuation moment, the molecular relaxation time constants, and the chemical rate constants of the ionic binding reaction. Contrary to a previous treatment of this problem by Kirkwood and Shumaker [21], Scheider [22] showed that fluctuations introduce no independent components into the relaxation spectrum.

In my first experiments with the cell that I borrowed from Prof. Scatchard and before I started the carboxyhemoglobin work I discovered that there was a large polarization capacity to deal with whenever I tried to study dilute aqueous solutions. This had introduced a correction in conductivity measurements, and capacitance measurements showed a considerably larger effect. Conductivity measurements had shown that this involved a term with a fractional power dependence, usually found to be near $1/2$ which might better be called a 'pseudo-capacitance' since by definition a capacitance is frequency invariant. Scheider showed that the surfaces of electrodes used in these cells has a microscopic surface roughness, and must be looked on as branched ladder networks with distributed properties which lead to this fractional power of the frequency [23].

10. What dielectric constant studies have contributed to our knowledge of serum albumin structure

When I first undertook my dielectric studies of hemoglobin and serum albumin in 1936 my aim was to show experimentally the anomalous dispersion predicted by Peter Debye. In my 1932 Ph.D. dissertation I tried to do this by studying nitrobenzene and nitronaphthalene dissolved in high viscosity hydrocarbon solvents and when those solutions failed to show dispersion I moved to what I imagined would be very large protein molecules of completely rigid character. When that changed my career from the study of electrical insulation to the study of proteins, the use of Perrin's equations allowed a study of rotary Brownian motion by dispersion of the dielectric constant. I again found that the data failed us. It seems that additional relaxation times for internal rearrangements of the albumin after ligand binding are in the dispersion of the dielectric constant of serum albumin, and may be confused with the Perrin relaxation times due to the rotary Brownian motion. There may also be difficulties in extrapolation of concentration effects to low albumin concentration, and experimental difficulties in extrapolation to zero concentration that caused the prediction of an elongated ellipsoidal shape for the albumin molecule.

But once again, the dielectric studies of Scheider have proved important in allowing a detailed stop–flow kinetic study of the fatty acid interaction of a highly specialized protein structure. In serum albumin the amino acid sequence determines not only the shape of the final albumin molecule, but that of numerous multiligated molecules—at least five, and probably many more.

I would like to acknowledge the help of Walter Scheider and Howard Dintzis in the preparation of this review, of Millie Johnson in checking the references, and of Jennifer Thyng with helping to keep my computer going.

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