to the molar refraction R_D as calculated from bond refractions values.[29]

(29) A. I. Vogel, W. I. Creswell, G. H. Jeffery and J. Leicester, *J. Chem. Soc.*, 514 (1952).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, SCHOOL OF MEDICINE, UNIVERSITY OF PENNSYLVANIA]

Dielectric Properties of Hemoglobin. VI. Measurements with Solid Materials

BY SHIRO TAKASHIMA

RECEIVED DECEMBER 23, 1957

The dielectric increments and the dispersions of horse oxy- and carboxyhemoglobin in the frozen state were measured between −5 and −40° in a frequency range of 400 kc. to 18 mc. A considerable decrease of the dielectric constants of both hemoglobins were observed on freezing the solution. The dielectric increments per gram at −5° are 0.10 and 0.02 for oxy- and carboxyhemoglobin, respectively. The dielectric constant of oxyhemoglobin crystals was measured in the same temperature and frequency range. Analysis of the anomalous dispersion and its temperature dependence was based on the results obtained with the crystal sample. It was found that the dielectric polarization of oxyhemoglobin passes through at least three consecutive stages with decreasing temperature. The dipole moments obtained were in good agreement with the theoretical value of Kirkwood.

Introduction

The dielectric polarization of protein molecules has been explained by the orientation of the whole molecule in the electric field. Thus, Oncley's[1] results on the anomalous dispersion of hemoglobin solutions were successfully interpreted by means of the rotary diffusion theory. However, recently Jacobsen[2-4] suggested that orientation of the whole molecule is not likely to occur in the dielectric polarization of protein solutions and that excitation of the hydrated water molecules is the substantial mechanism of the polarization. Similarly, Bayley[5] stated that the polarization of protein crystals increases in the presence of sorbed water. On the basis of dipole moment calculations, Kirkwood[6] suggested that the induced moment, which he attributed to fluctuation of mobile protons, is the major part of the dipole moment of some protein molecules, including hemoglobin.

The experimental approach to this problem is difficult. A rather indirect experiment of Timasheff, *et al.*,[7] on the charge fluctuation of albumin by the light-scattering method indicates that the dipole interaction of albumin molecules accompanies the fluctuation of 3.58 protonic units per molecule.

Therefore, we have studied wet hemoglobin crystals and also frozen samples in order to observe the polarizability in the presence of sorbed water. The results have been analyzed thermodynamically and discussed in terms of the dielectric theories concerning protein molecules.

Experimental

The bridge substitution method was used for measurement of the dielectric constants. Since the capacity and conductivity of ice are very small in the radiofrequency range, the Twin T bridge which is suitable for the measurement of the large impedance, was used. The frequency in this experiment is high enough to be outside the dispersion region of ice. Therefore, it behaved like a nonpolar substance and had a dielectric constant of about 4 which is favorable for the observation of the dielectric increment. When water is used as the solvent, the small increase of dielectric constant due to the protein molecules is difficult to detect because of the high conductivity and large dielectric constant of water itself. However, since in our case the dielectric increment has the same order of magnitude as the dielectric constant of the ice, the measurements are very precise. The dielectric constant of ice is almost independent of the temperature at these frequencies and should have a constant value throughout the radio-frequency range. However, the bridge does not function properly at high frequency and a slight decrease of capacity was always observed above 10 megacycles. The dielectric increment of protein at each frequency was obtained by subtracting the dielectric constant of ice at the same frequency from the total dielectric constant. Thus the total dielectric increment of the protein solid is given by the following equation; $\Delta\epsilon_t = (\epsilon_t - \epsilon_i)_0 - (\epsilon_t - \epsilon_i)_\infty$ where ϵ_t and ϵ_i are the total dielectric constant and the dielectric constant of ice, respectively, and $(\epsilon_t - \epsilon_i)_0$ and $(\epsilon_t - \epsilon_i)_\infty$ are the dielectric constant of protein at low and at high frequencies.

Because of the very small dielectric constant of the material, the cell design and the effect of connecting leads are important. Precautions were taken to minimize the effect of stray capacity and an electrode distance was selected within the region in which the capacity bears a linear relation to the electrode distance. The electrodes were plated with platinum black and practically no electrode polarization was observed in this frequency range. The electrodes were completely enclosed in the frozen material.

The temperature was varied from −4 to −40°. The protein solution (15 to 25 g. per liter) was frozen quickly by immersion of the solution cell in Dry Ice–acetone. The capacity readings were repeated until a constant value was obtained. The temperature control was far from ideal. Since the measurement over the entire frequency range takes considerable time, some heat exchange was unavoidable even though the cell was enclosed in a Dewar flask.

Results

The dielectric constants of frozen horse oxyhemoglobin are shown in Fig. 1, in which $\epsilon_t - \epsilon_i$ is plotted against the logarithm of the frequency in mc. It is obvious that the protein solid shows anomalous dispersion in nearly the same frequency range as the solution. The temperature dependence of the dielectric increments and of the relaxation time are plotted in Figs. 2 and 3,[8] respectively.

(1) J. L. Oncley, THIS JOURNAL, **60**, 1115 (1938).

(2) B. Jacobsen, *Rev. Sci. Instr.*, **24**, 10, 249 (1953).

(3) B. Jacobsen and M. Wenner, *Biochim. Biophys. Acta*, **13**, 577 (1954).

(4) B. Jacobsen, THIS JOURNAL, **77**, 2919 (1955).

(5) S. T. Bayley, *Trans. Faraday Soc.*, **47**, 5, 509 (1951).

(6) J. Kirkwood and J. B. Schumaker, *Proc. Nat. Acad. Sci.*, **38**, 855 (1952).

(7) S. N. Timasheff, H. H. Dintzis, J. Kirkwood and B. D. Coleman, THIS JOURNAL, **79**, 782 (1957).

(8) The values for the temperature above the freezing point are taken from the previous paper, to be published in *Arch. Biochem. Biophys.*

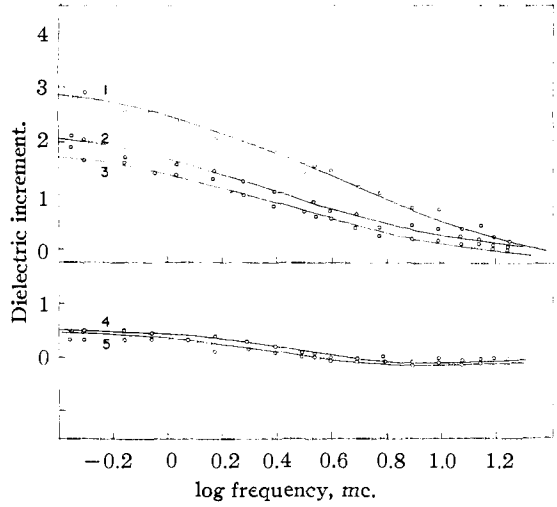

Fig. 1a and b.—The dielectric constant of frozen oxyhemoglobin solution and its dispersion. Curves 1, 2, 3, 4 and 5 were obtained at −5, −10, −20, −30 and −40°, respectively.

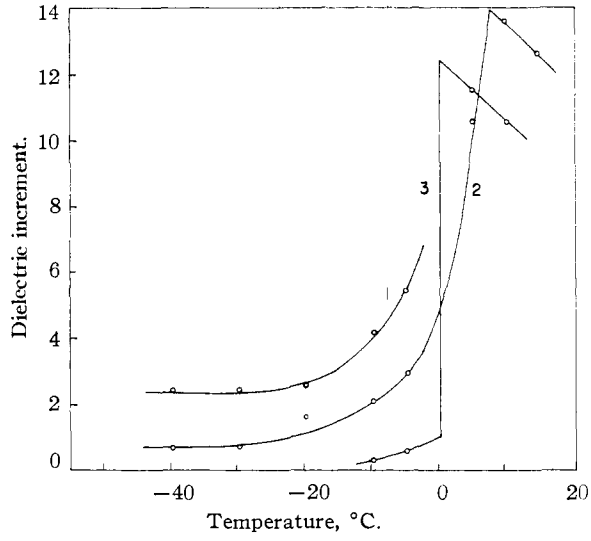

Fig. 2.—The temperature dependence of the dielectric increment of hemoglobin solids: curves 1, wet oxyhemoglobin crystal; curve 2, frozen oxyhemoglobin solution; curve 3, frozen carboxyhemoglobin.

The results of comparable measurements with wet crystals are given in Fig. 4; the temperature dependence of the dielectric constant and its dispersion are more clearly shown with the crystal sample. The temperature dependence of the dielectric increment and the relaxation time are included in Fig. 2 (curve 1) and Fig. 3 (curve 1), respectively. The temperature dependence of the dielectric constant of the crystal is similar to that of the frozen solution except that the values are higher. The relaxation time, however, undergoes a more drastic change between −20 and −30° than that of the frozen solution.

It is noteworthy that the transition between −5 and −30° is accompanied by a considerable change in the shape of the dispersion curve. The normalized dispersion curves at −5 and −30° are presented in Fig. 5. As the figure shows, the dispersion

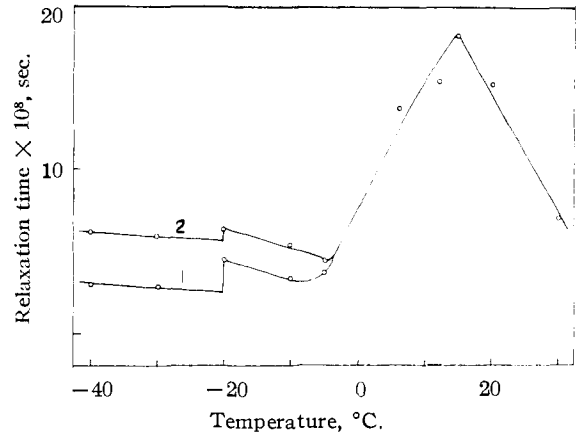

Fig. 3.—The temperature dependence of the relaxation time of hemoglobin: curve 1, oxyhemoglobin crystal; curve 2, frozen oxyhemoglobin solution.

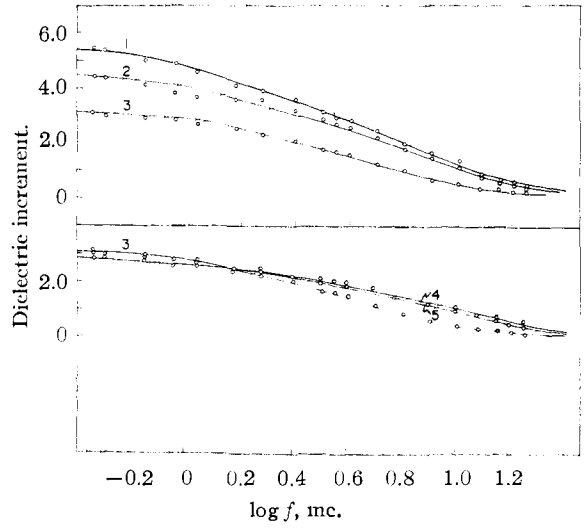

Fig. 4a and b.—The dielectric constant and its dispersion of wet oxyhemoglobin crystal. Curve 1, 2, 3, 4 and 5 were obtained at −5, −10, −20, −30 and −40°, respectively.

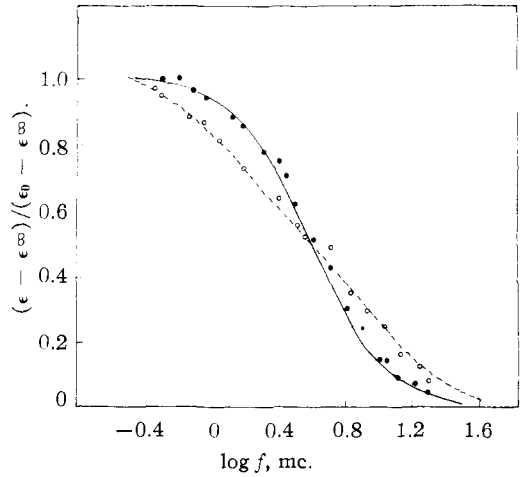

Fig. 5.—The normalized dispersion curve of hemoglobin crystal compared with the Debye dispersion curve. The solid line is the Debye dispersion; open circles are for −5° and closed circles for −30°.

at −5° deviates considerably from that predicted by the Debye theory, but the dispersion at −30° fits very well. This fact suggests that there is a transition of polarization mechanism between these temperatures.

Similar studies were made with carboxyhemoglobin (see Fig. 6a). As the dielectric constant of this protein is very small in the frozen state, it was difficult to determine the dielectric increment precisely. However, the increment was estimated and plotted as a function of the temperature in Fig. 2 (curve 3). In contrast to the dielectric increment of oxyhemoglobin, that of carboxyhemoglobin shows a rapid decrease at the freezing point and almost disappears at −10°.

The dielectric constant of urea (1 *M* solution frozen at 10°) was measured in the same frequency range. Urea has a dipole moment of 4.56 and should have a considerable dielectric increment in solution, but as Fig. 6b shows, no appreciable increment was observed with the frozen solid in the entire frequency range measured.

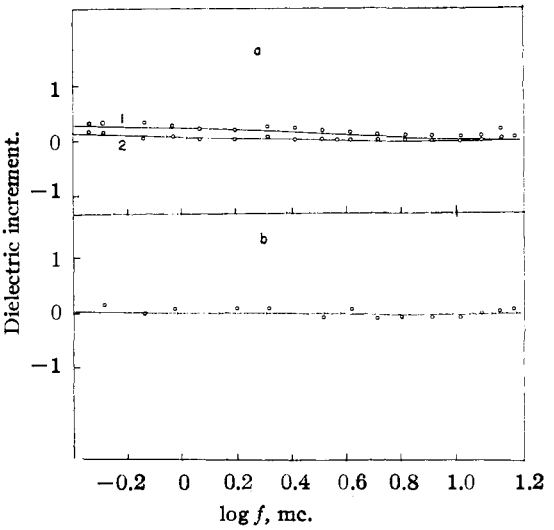

Fig. 6a.—The dielectric constant of carboxyhemoglobin solid: curve 1, −5°; curve 2, −10°.

Fig. 6b.—The dielectric constant of urea in frozen solution at −10°.

Discussion

The dielectric constant of oxyhemoglobin decreases considerably between 10 and −10° and shows no discontinuity at the freezing point of water. Apparently oxyhemoglobin molecules associate even above the freezing point of water and lose the freedom of orientation, a condition which results in the decrease of the dielectric constant. The decrease of the dielectric constant near the freezing point of the solvent strongly indicates that the dielectric polarization of this protein is largely due to orientational polarization. However, the dielectric increment does not show a sharp transition but decreases asymptotically to a lower plateau, and furthermore it does not disappear even at temperatures far below the freezing point; these facts indicate that a polarization mechanism, which may be associated with the charge displacement, is making a considerable contribution to the total polarization. It is noteworthy that the dielectric increment has a well-characterized anomalous dispersion in this frequency range which is greater than that of the solution. The different shape of the dispersion curve, smaller relaxation time and its temperature dependence suggest that the dielectric increment of the solid has different origin from that of the solution.

The transition of the polarization mechanism from solution to solid results in a change of dielectric increment, as mentioned above, and particularly in a change in the relaxation time. As shown in Fig. 3, the relaxation time passes through four consecutive stages with decreasing temperature. The relaxation time has a considerable temperature dependence between 30 and 15° which is due to the orientational polarization of the whole molecule. The following negative temperature dependence must be due to the phase shift. The intermediate stage between −5 and −20°, which may be partially obscured by the transition stage, appears to have a much smaller temperature dependence. The relaxation time decreases suddenly between −20 and −30° below which it is almost independent of the temperature.

The behavior of the dielectric increment and the relaxation time suggests that besides the orientational polarization, some polarization mechanism which may be associated with charge displacement, is making a considerable contribution to the total polarizability.

The behavior of carboxyhemoglobin is very different from that of oxyhemoglobin. The dielectric increment of this protein decreases very rapidly at the freezing point of water and almost disappears at −10°. This sharp order–disorder transition indicates that the polarizability of carboxyhemoglobin consists mainly of the orientational polarization and the contribution of charge displacement is negligible. Further investigation is necessary to explain the large difference between the dielectric properties of oxy- and carboxyhemoglobin.

The results on oxyhemoglobin strongly indicate that the dielectric increment of the solid sample must have an entirely different origin. Of the theories of the dielectric polarization of protein molecules, those of Kirkwood, *et al.*, and Jacobsen are relevant to the present experiment.

Kirkwood and Shumaker assumed that the dielectric polarization of the protein molecule is due to the fluctuation of mobile protons along the basic sites of the molecule in the direction of the electric field; they derived an equation for the dipole moment of protein molecules, in which they introduced, as the parameters the dissociation constants of basic groups, the shape of the molecule and the mean distance of basic sites from the center of the molecule.

According to their theory, the total dipole moment consists of the fixed dipole moment plus the dipole moment which is associated with the fluctuation of mobile protons as

$$\langle\mu^2\rangle_{ave} = \langle\mu\rangle^2_{ave} + \Delta\mu^2$$

where $\langle\mu\rangle_{ave}$ is the average fixed dipole moment and $\Delta\mu$ is the dipole moment due to the mobile pro-

tons. The magnitude of $\Delta\mu$ is given by the equations

$$\Delta\mu^2 = e^2 f^2 b_0^2 \Sigma \frac{\nu_\alpha}{2 + K_\alpha/[H^+] + [H^+]/K_\alpha}$$

$$f^2 = \frac{\sigma^{4/3}}{4} \frac{(\sigma^2 + 2)\sqrt{\sigma^2 - 1} + \sigma^2(\sigma^2 + 4)\sec.^{-1}\sigma}{\sigma^2\sqrt{\sigma^2 - 1} + \sigma^4 \sec.^{-1}\sigma}$$

$$\sigma = a/b \quad b_0 = (ab)^{1/3}$$

where e is the protonic charge and ν_α is the mean distance of the basic sites from the center of the molecule; it is assumed that all the basic sites are evenly distributed on the surface of the protein molecule. K_α is the dissociation constant of the basic groups and b_0 is the radius of a sphere of the same volume, a and b are the long and short axis of the molecule which is assumed to be an ellipsoid of revolution.

They calculated the dipole moment of hemoglobin for two cases: (1) in which the molecule is assumed to be a sphere and (2) in which it is assumed to be an ellipsoid of revolution with an axial ratio of 1:5. The results of diffusion and viscosity studies[9] indicate that (2) is the better approximation for oxyhemoglobin. They also carried out the calculation taking into account the electrostatic interaction between protons. The fact that the dispersion curves of the solid protein sample have a broad distribution suggests that there is a restricting interaction between protons. Furthermore, as will be seen, the value calculated on the basis of these is in better agreement with the experimental results, a finding which indicates the validity of this assumption.

The dielectric increment of oxyhemoglobin solid is 0.1 per gram at $-5°$, which leads to a dipole moment of about 200 debye calculated with Oncley's formula. However, the molecules are embedded randomly in the medium and their orientation is supposed to be strongly restricted by the almost infinite viscosity of the medium. Accordingly the observed moment is the mean value of the projection of the induced dipole moment of each molecule along the direction of the electric field. If it is assumed that the distribution of the directions of the vectors (regarded as that of points on the unit sphere) is uniform, we obtain the following relation between $\bar{\mu}$ and μ

$$\bar{\mu} = \mu \{\int |\cos\theta| \, ds + \lambda \int |\sin\theta| \, ds\}$$

Thus

$$\mu = \bar{\mu} \Big/ \frac{1}{2}\left(1 + \lambda \frac{\pi}{2}\right)$$

where $\bar{\mu}$ is the observed average moment and the μ is the dipole moment of the molecule and λ is the relative magnitude of the minor axis of the molecule.[10] Since the axial ratio is 1/5, the value of λ is 0.2, setting the value of major axis as 1. The results of the calculations are given in Table I which shows that the theoretical value is of the same order of magnitude as the experimental values. This agreement indicates that the dielectric increment of oxyhemoglobin solid may be due to mobile proton fluctuation.

TABLE I

COMPARISON OF THE OBSERVED DIPOLE MOMENTS OF OXYHEMOGLOBIN WITH DIPOLE MOMENT CALCULATED WITH KIRKWOOD THEORY

Temp., °C.	μexp	μcalcd.		
	Soln.	With electrostatic interaction	Sphere	Ellipsoid
15	525		300	480
10	545			
5	460	Without electrostatic interaction	390	620
	Solid			
− 5	345			
−10	262			
−20	225			
−30	200			
−40	200			

Bayley who investigated the dielectric properties of some proteins with dry crystals, found that the dielectric constants are of the order of 2 for the entire frequency range and have no characterized dispersion. He also reported that the dielectric constants of crystal samples are very dependent on the small amount of sorbed water. He attributed the increase of dielectric constant of wet crystals to the dielectric polarization of sorbed water, a conclusion which appears to be in harmony with Jacobsen's hypothesis. Furthermore, his theory states that the polar groups on the protein surface impose restriction on the rotation of the surrounding water molecules which results in anomalous dispersion at far lower frequencies than that of water itself.

Although this is a reasonable interpretation, we cannot ignore the possibility that the sorbed water increases the dissociation of polar groups of the protein surface and thus enhances the fluctuation of mobile protons. Unfortunately we do not have experimental evidence but the estimation of the potential barrier for the dielectric polarization of the solid sample may shed some light on the problem. The heat of activation, free energy of activation and the entropy change were calculated by Kauzmann's equations[11] which are based on the Eyring rate theory.

$$\tau = \frac{1}{k_0} = \frac{h}{kT} e^{-\Delta F^*/RT}$$

$$\Delta H^* = R \frac{\mathrm{d} \ln \tau}{\mathrm{d}(1/T)}$$

where τ is the relaxation time and k_0 is the rate constant of the relaxation process. The heat of activation was obtained from the temperature dependence of the relaxation time which is replotted in Fig. 7. Unfortunately only a few points are available for each portion of the curve. The thermodynamic quantities obtained are listed in Table II together with those of hemoglobin solution, water and ice.

As is evident from the table, the heat of activation of the dipole polarization of oxyhemoglobin solid is considerably smaller than that of the solution and even smaller than that of water. The small heat of activation results in the striking decrease of

(9) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943.

(10) Since the induced moment should depend only on the dimension of the molecule and not on the permanent dipole moment, λ is taken as the shape factor.

(11) W. Kauzmann, *Rev. Mod. Phys.*, **14**, 12 (1942).

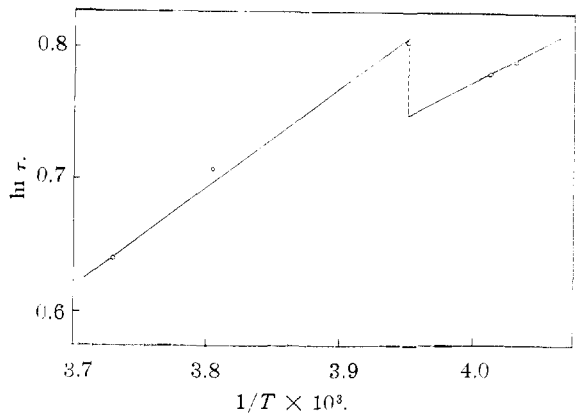

Fig. 7.—The temperature dependence of relaxation time of oxyhemoglobin crystal, ln τ *vs.* $1/T$.

entropy although the entropy decrease in the solution is small. The entropy change of the dielectric polarization may be considered to be the sum of the increase of the breaking of the directed valence bonds of solvent molecules and the decrease due to the orientation of the molecules in the direction of the field. But the orientation of the whole molecule is unlikely in the solid sample and the polarization may occur in such a way that it does not break the directed hydrogen bonds of water.

The marked difference between the thermodynamic quantities of the solid and solution supports our conclusion that the dielectric increments have different origins. However, it is unlikely that the dielectric increment of the protein solid has the same mechanism as that of ice, since the heat of activation of the protein solid is much smaller than that of ice and the entropy change very different. This fact eliminates the possibility of the polarization of hydrogen bonds by simple elastic displacement of protons in protein molecules and their environments.

TABLE II

THERMODYNAMIC QUANTITIES OF DIELECTRIC POLARIZATION OF HEMOGLOBIN

	ΔH^*, kcal.	ΔF^*, kcal.	ΔS^*, cal.
O_2Hb(crystal)	2.74	6.49	−14.3
COHb(soln.)[a]	5.56	7.80	− 7.7
Water[b]	4.05	2.30	5.67
Ice[c]	13.2	9.0	18.1

[a] S. Takashima, to be published in *Arch. Biochem. Biophys.* [b] C. P. Smyth, "Dielectric Behavior and Structure," Chap. 4, McGraw-Hill Book Co., Inc., New York, N. Y., 1955. [c] S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co., Inc., New York, N. Y., 1941.

These complex phenomena can only be explained by the assumption that at least two polarization mechanisms are involved in the dielectric behavior of protein solution and solid. Probably the presence of various polar groups and their coördination to water molecules makes the dielectric properties of protein molecules very complicated. However, information on the dielectric losses is not available for the protein solids. Thus, we lack the experimental background necessary for further analysis of the dispersion of the solid sample.

It is a pleasure to thank Dr. H. Yamabe of the University of Minnesota and Dr. H. P. Schwan for their advice. Also the author is indebted to Prof. H. Tamiya of the Tokyo University for his encouragement and guidance.

PHILADELPHIA, PENNA.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, SCHOOL OF MEDICINE, UNIVERSITY OF PENNSYLVANIA]

Dielectric Properties of Albumins

BY SHIRO TAKASHIMA

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The dielectric properties of egg and human serum albumins were studied with frozen solutions between −5 and −60°. Freezing of the solution results in a decrease of dielectric increment, a finding which indicates that the dielectric polarization of these proteins is largely due to the orientational polarization. However, these frozen solutions have small but well-defined dielectric increments. The dielectric polarization at low temperature was discussed in terms of the Kirkwood theory.

Introduction

The experiments with hemoglobin, described in the preceding paper, were extended to egg and human serum albumins, whose dielectric properties in solution were investigated by Oncley, *et al.*[1] The dipole moments of egg and serum albumin are about 280 and 700 debye, respectively. However, the dielectric increment of serum albumin obtained by Bayley[2] with dry crystals is extremely small.

Our results with frozen albumin solutions are essentially the same as those obtained with oxyhemoglobin. The dipole moments were calculated and compared with the theoretical values of Kirkwood.[3]

Experimental

Measurements were made as described in the preceding paper. Commercial egg and human serum albumins were used without purification, except that the solutions were dialyzed overnight before use. The concentration of protein was about 20–25 g. per liter.

The dielectric constants of egg and serum albumin and the anomalous dispersions are shown in Figs. 1 and 2. The dielectric increments per gram are 0.11 and 0.13 for egg and serum albumin, respectively, at −5°; the corresponding values at −20° are approximately 0.05 and 0.06. The

(1) J. L. Oncley, J. D. Ferry and J. Shack, *Ann. N. Y. Acad. Sci.*, **40**, 371 (1940); J. Ferry and J. L. Oncley, THIS JOURNAL, **60**, 1123 (1938).

(2) S. T. Bayley, *Trans. Faraday Soc.*, **47**, 5, 509 (1951).

(3) J. Kirkwood and J. B. Schumaker, *Proc. Nat. Acad. Sci.*, **40**, 371 (1940).