[CONTRIBUTION FROM BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY]

Dielectric Studies on Muscle Hemoglobin

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Hemoglobin (blood hemoglobin) and myoglobin (muscle hemoglobin) offer an interesting comparison. Both play their role as mechanisms for providing oxygen to the tissues, and in both the reversible combination with oxygen is due to the presence of the same prosthetic group, ferroheme, conjugated with a globin. They have closely similar absorption spectra.[1] Myoglobin is now known to have a molecular weight of about 17,000 and to contain only one heme.[2,3] In distinction from this, hemoglobin has a molecular weight of 68,000, or four times as much, and contains four hemes. There is evidence that these four hemes are identically related to identical portions of the globin.[4] From these facts it might be supposed that hemoglobin is a polymer of four myoglobin units. In further support of this, it is suggestive to observe that in concentrated urea solutions hemoglobin dissociates reversibly into two equal parts, although so far it has been impossible to obtain splitting into quarter size molecules.[5] On the other hand, there is evidence that this interpretation cannot be strictly true, for the effect of hydrogen ion concentration on the oxygen equilibrium is very much less for myoglobin than for hemoglobin.[6,7] This indicates that the acid groups in the globin which interact with heme are different in the two proteins. Nevertheless, if we concede at least some differences in the globins, it is legitimate to think of the structure of hemoglobin in this way.

From this point of view, a comparison of the dielectric behavior of the two proteins is of interest, besides being of some importance on its own account. Reliable data on the dielectric constant of hemoglobin are now available, but data for myoglobin are entirely lacking. Oncley's bridge measurements of solutions of blood carboxyhemoglobin give the real part of the dielectric constant over a frequency range from 0.025 to 2.50 megacycles with a very considerable degree of accuracy, and are supplemented by resonance measurements of one of us at about 75 megacycles.[8] These results agree with the classical Debye dispersion curve within the experimental error and lead to a critical frequency of 1.9 megacycles and a dielectric increment per gram of 0.33 for all solutions studied. The critical frequency agrees well with that calculated by Stokes' law for the hydrated hemoglobin molecule regarded as a sphere (2.1 megacycles). The question at once arises whether the molecules of myoglobin likewise conform to the Debye curve and behave like spheres, whether they have the shorter relaxation time required by their lower molecular weight, and whether they show the same dielectric increment per gram as hemoglobin. If hemoglobin were to be thought of as constituted of four units each similar to a myoglobin molecule and possessed of the same electric moment, these might be elongated or flattened and yet fit together to give a molecule having the properties of a sphere.[8a] If there were a considerable degree of stabilization of these units with electric moments parallel and pointing in the same direction, the polarization, and consequently the dielectric increment, per gram of hemoglobin should be greater than that of myoglobin. In the limiting case of complete parallel stabilization the effect should be a four-fold increase. On the other hand, if the four units were oriented independently or arranged at random as would be suggested by the behavior of synthetic peptides,[9] the dielectric increment per gram would be the same for myoglobin as for hemoglobin. The magnetic studies of Coryell and others[10] show that the magnetic moments of

(1) H. Theorell, *Biochem. Z.*, **268**, 55 (1934).
(2) T. Svedberg, *Kolloid-Z.*, **85**, 119 (1938).
(3) J. Roche and H. Vieil, *Compt. rend.*, **210**, 314 (1940).
(4) J. Wyman, Jr. and E. N. Ingalls, *J. Biol. Chem.*, **139**, 877 (1941).
(5) J. Steinhardt, *ibid.*, **123**, 543 (1938).
(6) R. Hill, *Proc. Roy. Soc.* (London), **B120**, 472 (1936).
(7) H. Theorell, *Biochem. Z.*, **268**, 64, 73 (1934).
(8) J. L. Oncley, THIS JOURNAL, **60**, 1115 (1938).
(8a) It should be noted that other results do not accord so well with the assumption that the hemoglobin molecule is a sphere. Values of the linear diffusion and sedimentation constants give a dissymmetry factor in the neighborhood of 1.16. To account for this entirely on the basis of hydration it would be necessary to assume 0.42 g. of water per gram of protein. Results on viscosity would require a still higher figure. On the basis of all available data Oncley has concluded that about the best compromise is to assume 30% hydration and an axial ratio of 1.6 for the hemoglobin molecule regarded as a prolate ellipsoid of revolution. See J. L. Oncley, *Annals of New York Acad. of Sciences*, **XLI**, 121 (1941).
(9) J. Wyman, Jr., *J. Phys. Chem.*, **43**, 143 (1939).
(10) C. D. Coryell, L. Pauling and R. W. Dodson, *ibid.*, **43** 7 (1939).

the four hemes in blood hemoglobin behave as if arranged essentially at random although the accuracy of their results would not preclude a small degree of stabilization.

A study of the dielectric properties of myoglobin presents considerable difficulties. For one thing, the protein is much less stable than blood hemoglobin and much more difficult to obtain in quantity. Also, in view of the size of the molecules it is necessary to extend the measurements to frequencies at least four times as great as in the case of hemoglobin. In view of these considerations the oscillograph method described in an earlier study is particularly well suited. This permits very rapid measurements on only 2 ml. of solution as opposed to about 25 ml. required by the bridge method used by Oncley. It may be extended to much higher frequencies and has the advantage that it yields both the real and imaginary components of the dielectric constant.

In the present study we have used this method for the investigation of ferrimyoglobin over a range of frequencies from 1.76 to 15 megacycles (λ = 170 to 20 meters). We used the oxidized form since the protein undergoes autoxidation during the preparation of the final dialyzed material.

Preparation of Myoglobin

The myoglobin crystals were prepared according to the procedure of Theorell.[11] Minced horse heart was extracted with half its weight of water for twenty-four hours at 2–4°. The dark red filtrate was then separated from the mince by centrifuging. The *p*H was adjusted to 7.0, with the aid of a glass electrode, by adding normal sodium hydroxide. Solid basic lead acetate, $Pb(Ac)_2Pb(OH)_2$, to the extent of 5.5% by weight of the original mince, was then added with stirring at room temperature. After removal of the precipitated muscle globulins and other impurities and readjustment of the *p*H to 7.0, the remaining lead acetate was precipitated with an equimolar mixture of diphosphate and monophosphate. Care was taken to stir the mixture gently since otherwise the fine precipitate adsorbed nearly all the colored matter in the filtrate. After removal of the voluminous precipitate, the *p*H was again adjusted to 7.0. The resulting clear dark red solution (about 950 ml.) was dialyzed against neutralized saturated ammonium sulfate for twenty-eight hours until the specific gravity was 1.197. The precipitate, although containing some myoglobin crystals, was discarded. Continuation of the dialysis to completion yielded the bulk of the myoglobin as a dark reddish brown paste. This was washed twice with saturated ammonium sulfate and examined under the microscope. Rod-shaped crystals, typical of myoglobin, were the principal matter observed and only a small amount of amorphous substance was present. The yield as determined spectroscopically was 0.7 g. per kilogram of minced heart. The paste was redissolved in 150 ml. of water and recrystallized by dialysis as before.

In the preparation it is of the utmost importance to obtain hearts of freshly killed horses and to proceed as rapidly as possible through the addition of lead acetate to destroy enzymes present in the minced heart.[11] In the present case the horse heart was cooled with ice, perfused with cold physiological saline solution, and minced within two hours after the death of the horse. Extraction was begun immediately with a mixture of ice and water. The lead acetate was added to the filtrate (*p*H 7.0) twenty-eight hours after the horse was killed. The first crystals were obtained six days later. The final crystals, obtained nine days after the horse was killed, were dissolved in water and dialyzed to give the solution studied.

Since a very low d. c. conductivity is required for the electrical measurements, great pains were taken to remove all salt contaminants from the final solution of the myoglobin crystals. This was accomplished initially by dialyzing the solution, contained in a small cellophane sac, against running distilled water for four days, and, finally, to remove the last traces of salt, by electrodialysis in a three compartment cell with platinum electrodes. The middle compartment (15 ml.), containing the myoglobin solution, was separated from the other two compartments, containing the electrodes, by thin cellophane membranes. Running distilled water was passed through the electrode compartments during the electrodialysis. This was continued for two days with an applied potential of 200 volts. At the conclusion of this procedure the specific conductivity of a 2.3% solution of myoglobin was only 16×10^{-6} mho. Some myoglobin was denatured by the electrodialysis and this was removed by centrifuging to yield 6 ml. of clear, dark red solution. By this time the protein was all in the oxidized form.

The volume of this salt-free solution was made up to 8 ml. with twice distilled water. Of this 4 ml. was used to determine the dry weight of the solution, the nitrogen content, the spectroscopic absorption, the *p*H, and the viscosity. The remaining 4 ml. was divided equally and used to make two sets of dielectric constant measurements at seven frequencies.

The concentration, determined by drying 0.6 g. and 0.9 g. aliquots to constant weight at about 100°, was found to be 2.31 ± 0.02 weight per cent. A micro-Kjeldahl determination on a diluted aliquot gave the nitrogen content as 3.63 mg. per gram of original solution. The latter figure, on the basis of Theorell's value[12] of 16.6% for the nitrogen content of dry myoglobin, gives a concentration of 2.18 weight per cent. The discrepancy between this and 2.31, amounting to about 6%, is probably to be ascribed to errors in the Kjeldahl rather than to impurities in the solution. The figure 2.31, obtained by drying, is to be preferred and is the one we use.

As a further check on the purity of the preparation, the absorption spectrum was studied with a grating type of photoelectric spectrophotometer made by the Central Scientific Company. Two dilutions of the original solution were measured, with a mercury vapor lamp as light

(11) H. Theorell, *Biochem. Z.*, **252**, 1 (1932).

(12) H. Theorell, *ibid.*, **268**, 64 (1934).

source. The breadth of the band employed was never more than 6.5 mμ and for the most part less. In the region of the bright mercury lines it was between 2.9 and 3.3 mμ. The results, for *p*H 6.1, are plotted in Fig. 1 beside the curves given by Theorell[13] for *p*H 6.2 and 7.0. Except

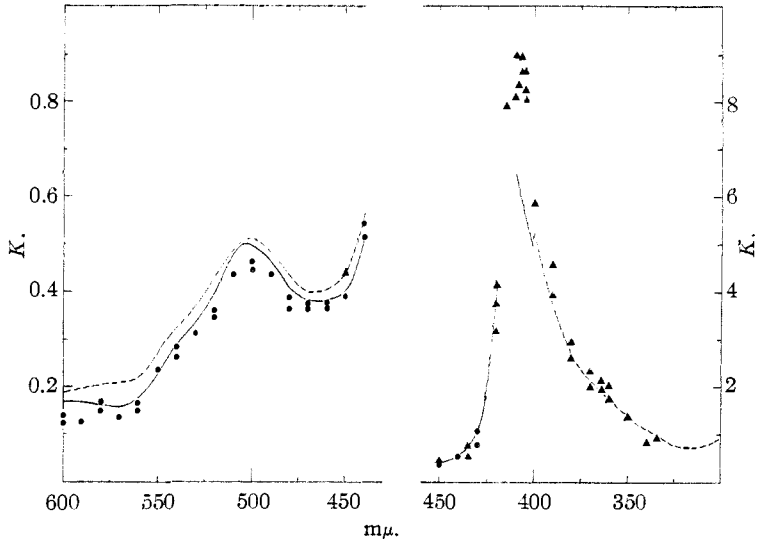

Fig. 1.—The absorption coefficient K of ferrimyoglobin in aqueous solution in relation to wave length, mμ. The circles are for a concentration of 0.718 g. per liter; the triangles for a concentration of 0.0881 g. per liter. The full curve gives Theorell's results obtained by the Warburg method at *p*H 6.2; the broken curve his results at *p*H 7.0, obtained by both the Warburg and photographic methods.

near the absorption peak at 407 mμ the agreement between our results and Theorell's curve for *p*H 6.2, obtained with the Warburg method, is satisfactory in view of the differences of method and the experimental errors. These errors, as judged by the discrepancies shown by Theorell's two sets of measurements for *p*H 7, one made with the Warburg, the other with a photographic, method are of the same order as the discrepancies between our points and Theorell's curve for *p*H 6.2. The much greater discrepancy at the absorption peak might be explained by our use of an unusually narrow slit in this region, made possible by the very intense mercury line at 408 mμ.

The *p*H was determined spectroscopically by adding a few drops of brom cresol purple to a dilute aliquot containing 0.0051 gram of myoglobin per liter. The absorption was measured at a wave length of 580 mμ before and after addition of 1 drop of 0.1 sodium hydroxide to the diluted solution. On the basis of preliminary measurements of the absorption of the pure acid and basic form of the dye, it is a simple matter to reckon the ratio of the two forms of the dye after correcting for the absorption due to the protein. Taking the pK of the indicator as 6.3,[14] we thus obtained 6.1 as the *p*H of the muscle hemoglobin solution measured.

In calculations of the relaxation time viscosity data are necessary. The viscosity of the solution was determined by means of an Ostwald viscosimeter at 25° and found to be 0.00960 poise. This figure involves a knowledge of the density, which was estimated to be 1.003 on the basis of the apparent specific volume 0.741 given by Theorell[15] for the salt-free protein at 20°.

The Method

The cathode ray oscilloscope employed in previous studies[16] was used to compare the impedances of two similar cells, one filled with water and the other with an aqueous solution of the protein. In this way both the real and imaginary components of the unknown impedance corresponding to the ordinary dielectric constant and to the power absorption, respectively, are determined with considerable accuracy.

The real part of the dielectric constant, ϵ', is given by the relation

$$\frac{\epsilon'}{\epsilon_s} = \frac{C_{so}}{C_{xo}}\gamma D\left\{1 - \frac{1.8 \times 10^{12} \tan\psi\, K_s}{\nu\epsilon_s}\right\}\lambda \cos\psi \quad (1)$$

Here ϵ_s and K_s are the dielectric constant and the specific conductivity respectively of the standard liquid; C_{so} and C_{xo} are the air capacities of the cells containing standard and unknown solutions, respectively; γ is a correction factor dependent on the parallel circuit formed by the oscillograph deflection plates together with the associated leads; D is a factor dependent on the geometry of the cathode ray tube; and ν is the frequency. λ and ψ, the principal observed quantities, are, respectively, the slope of the major axis of the elliptical oscillograph pattern with reference to the horizontal line generated by the vertical deflection plates, and the difference between the phase angles of the two impedances composed by the two cells. ψ is ordinarily determined by adjusting the transit time between the two sets of deflection plates to compensate for the phase difference between the potentials applied, a condition easily recognized by the degeneration of the elliptical oscillograph trace to a straight line. The term $1.8 \times 10^{12} \tan\psi K_s/\nu\epsilon_s$ represents the small correction resulting from the conductivity of the water in the standard cell.

The ratio of the imaginary component, ϵ'', to the

(13) H. Theorell, *Biochem. Z.*, **268**, 55 (1934).

(14) W. M. Clark, "The Determination of Hydrogen Ions," Williams and Wilkins Co., Baltimore, Md., 1928.

(15) H. Theorell, *Biochem. Z.*, **268**, 46 (1934).

(16) H. Marcy and J. Wyman, Jr., THIS JOURNAL, **63**, 3388 (1941).

real component of the dielectric constant is given by

$$\frac{\epsilon''}{\epsilon'} + 1.8 \times 10^{12} \left(\frac{K_x}{\nu\epsilon'} - \frac{K_s}{\nu\epsilon_s} \right) = \tan \psi \qquad (2)$$

where K_x and K_s are, respectively, the low frequency conductivities of the unknown and standard solutions. This equation assumes that $1.8 \times 10^{12}(K_s/\nu\epsilon_s) \sin \psi \ll \cos \psi$. Both eqs. (1) and (2) neglect the effect of the inductance and resistance of the short leads to each cell, an assumption justified by control experiments on model solutions containing potassium chloride and glycine.

It is apparent that the conductivity of the standard solution should be kept low in order to make eq. (2) valid to the lowest frequencies necessary to cover the dispersion range. Likewise K_x should be kept low, first so that ϵ''/ϵ' may not reduce to a second order effect; second, so that ψ may be small and thus reduce the error in $\cos \psi$ and $\tan \psi$ in equations 1 and 2 for a given error in ψ. It will be appreciated readily that the adverse effect of conductivity increases with decreasing frequency.

The oscillograph patterns were photographed to obtain a projection on a plane surface and thus simplify the measurements. This also permits enlargement of the pattern, eliminates possible distortion of the trace from the magnetic or electric fields associated with the measuring instruments or the hand, and reduces the time required for observations. In cases where the oscillograph pattern could not be reduced to a straight line the phase difference, as shown by the oscillograph trace, was easily calculated from the distance between the intercepts of the ellipse on the horizontal line generated by the vertical deflection plates and the total horizontal projection of the ellipse. The $\sin^{-1}$ of the ratio of these distances gives the phase difference in radians.

The source of alternating current was a tri-tet crystal oscillator followed by frequency doubler and amplifier stages. Three crystals calibrated to 0.05% and constant to about 0.05% were used interchangeably. The frequency doubler or tripler stages could be used when current of two or three times the fundamental frequency of the crystal was desired. In this way seven conveniently spaced frequencies from 1.76 to 15 megacycles were obtained from the three crystals.

The cells consisted of two holes drilled in a block of pure tin with cylindrical inner electrodes, also machined from pure tin, separated from the block by lucite plugs at the bottom. These inner electrodes were bent at right angles above the block and fastened to a covering slab of lucite. The latter was drilled with holes corresponding to the holes in the tin block, thus permitting the solution to extend above the tin block and in so doing to enclose most of the electric field. The whole cell was rigidly fastened with duco cement. Pure tin was employed to reduce the conductivity of the solutions to the lowest value possible.

Since the two experimental cells could not be made identical, calibration was necessary to determine the ratio of the capacities of the cells in air. The correction for this may be lumped with the other corrections γ and D in eq. (1), and the combined correction determined as a single factor by filling both cells with water and measuring the slope of the major axis of the ellipse. In this case ϵ'/ϵ_s, $\cos \psi$ and the factor in braces reduce to unity.

K_s and K_x may be determined directly from low-frequency bridge measurements of the same cells. Moreover, the ratio of the low-frequency admittances of the two cells filled with the same liquid gives the ratio of their air capacities, C_{so} and C_{xo}, and makes it possible to evaluate γD in eq. (1). Since D depends entirely upon geometrical factors, it may be calculated and thus the electrical constant γ determined. When this is done it is found that γ shows no frequency dependence. This means that we may neglect the inductance and resistance of the leads to the cathode ray tube.

Temperature regulation was accomplished by keeping the cell block immersed in a water-bath at 25° during the measurement of the conductivities. For the dielectric measurements it was necessary to remove the cell from the bath. The time taken to photograph the oscillograph pattern was, however, so short (about 20 sec.) that no appreciable change in temperature took place.

The entire run allowing for two observations at each of the seven frequencies required a matter of six hours. Nevertheless, the values at the beginning and at the end of the run checked well within the experimental error. This would indicate that the protein did not undergo any significant denaturation during the measurements.

The Results

The experimental data are given in Table I. Each value is the result of averaging four ob-

TABLE I

2.3% AQUEOUS SOLUTION OF MYOGLOBIN AT 25°

Freq. mc.	λ Degrees	ψ Degrees	$K_{MHb} \times 10^6$	$K_{H_2O} \times 10^6$	ε′	ε″
1.76	40.34	11.6	18.4	3.4	81.46	1.41
2.21	40.14	9.7	18.6	3.5	81.64	1.58
3.52	39.43	7.4	18.4	3.4	80.34	2.73
4.41	39.29	5.6	18.9	3.5	80.34	1.48
5.27	38.98	5.4	18.6	3.5	79.55	2.39
7.49	38.79	4.5	18.3	3.3	79.19	2.56
14.98	38.13	2.4	18.2	3.0	77.57	1.38

servations. An estimate of the accuracy of the results may be gathered, first, from the consistency of the separate observations and, second, from experiments with model solutions of potassium chloride and glycine. The ordinary dielectric constant shows a consistency of ± 0.5%. The agreement between the observed and calculated values for the model solutions was within 0.3%.

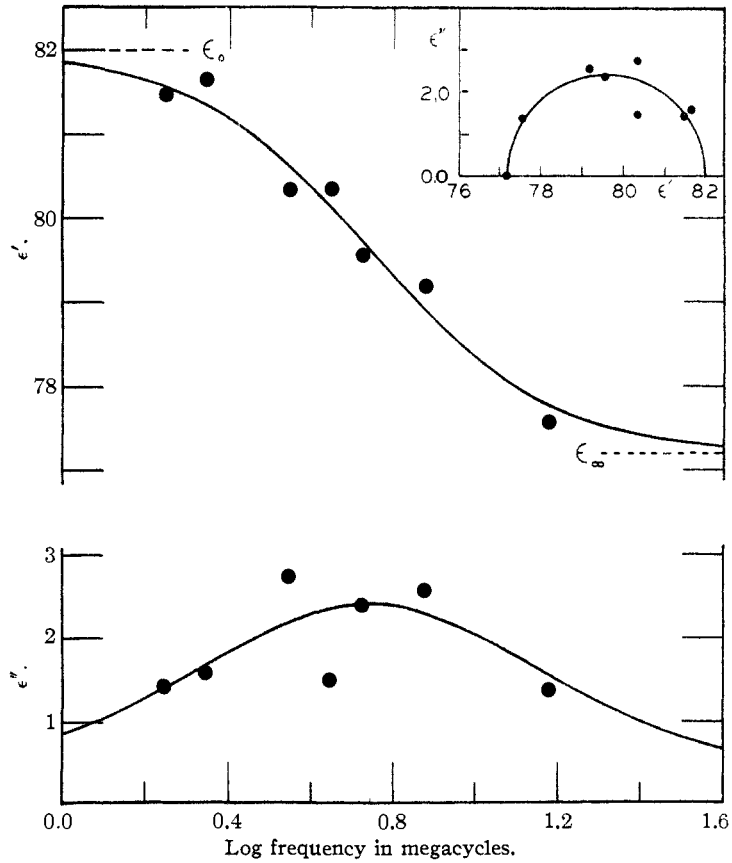

Fig. 2.—Real and imaginary parts of the dielectric constant of a 2.31% aqueous solutions of ferrimyoglobin in relation to frequency. Smooth curves are calculated from the Debye theory for a critical frequency of 5.54 megacycles.

The errors in the imaginary component of the dielectric constant, ε″, are much greater, particularly at the lower frequencies where the d. c. conductivity contributes very much more to ψ than does ε″. The results of studies of model solutions and the consistency shown by the individual observations made on myoglobin solutions both indicate deviations in ε″ decreasing from about ± 50% at a frequency of 1.76 mc. to ± 15% at 15 mc.

In Fig. 2, ε′ and ε″ are plotted against the logarithm of the frequency. The insert shows the Argand plot of ε″ against ε′. On the basis of the classical theory this plot should be a semicircle, and it is apparent that such is the case within the experimental error. The intercepts of this arc with the abscissa axis give the static dielectric constant, ϵ_0, and the high frequency dielectric constant, ϵ_∞, which are the asymptotes of the curve for ε′ plotted against log ν. It is also possible to determine the high frequency dielectric constant on the assumption that the volume occupied by the protein shows no appreciable polarization at frequencies far above that corresponding to the relaxation time. Using the figure 0.741 for the specific volume of myoglobin, ϵ_∞ is calculated to be 77.20. ϵ_0 determined by the use of the Argand plot is 82.0 ± 0.3. This gives a dielectric increment of 3.46 ± 0.30 for the 2.3% solution or an increment of 0.150 ± 0.013 per gram per liter.

With ϵ_0 and ϵ_∞ fixed it is possible to plot the classical Debye curves for ε′ and ε″ against log $x\nu$ from the equations

$$\epsilon' = \epsilon_\infty + \frac{\epsilon_0 - \epsilon_\infty}{1 + x^2\nu^2} \qquad (3)$$

$$\epsilon'' = \frac{(\epsilon_0 - \epsilon_\infty)x\nu}{1 + x^2\nu^2} \qquad (4)$$

where x is defined in terms of the critical frequency ν_c and the relaxation time τ by[17]

$$x = 1/\nu_c = 2\pi\tau$$

These curves, plotted on the same scales as the experimental points giving ε′ and ε″ against log ν, were superposed on the graph of the latter and shifted along the abscissa axis until the best fit between the curves and the points was obtained. The critical frequency was then identified as the intersection of the line log $x\nu = 0$ in the plot of the Debye

(17) J. Wyman, *Chem. Rev.*, **19**, 213 (1936).

curve with the abscissa axis on the experimental plot. The curves shown in Fig. 2 correspond to $\nu_c = 5.54$ megacycles or $\tau = 2.87 \times 10^{-8}$ sec. These values are accurate to within 12%.

Conclusions

It is evident from Fig. 2 that the data on myoglobin conform to the classical Debye theory within the rather large limits of error of the measurements. The critical frequency of 5.5 megacycles corresponding to the Debye curves drawn through the data should be compared with the value calculated by assuming that the molecules are spherical and applying Stokes' formula for a rotating sphere. If no account is taken of hydration, the critical frequency calculated in this way, on the basis of the viscosity and specific volume data already given, is 10.9 megacycles. The twofold discrepancy might be explained on the basis of hydration, shape, or a combination of the two. To account for it in terms of shape alone we may assume that the molecule is an elongated ellipsoid of revolution with the electric moment distributed principally along the major axis. In this case an application of Perrin's formula gives an axial ratio of 2.6. This may be compared with an axial ratio of approximately 2.8 calculated without assuming hydration by Eriksson-Quensel on the basis of the dissymmetry factor 1.1 obtained from the linear diffusion and sedimentation constants.[18] On the other hand, to account for the discrepancy wholly by hydration would demand 0.74 g. of water per gram of protein. Although no direct data are available on the hydration of myoglobin, this figure appears unreasonably large in view of what is known of other proteins, notably hemoglobin. The dissymmetry factor 1.1 would be completely accounted for by assuming 25% hydration. From these considerations the explanation in terms of shape would appear to lie closer to the mark. Nevertheless, there is evidence that most proteins are to some extent hydrated, and it is probable that the best explanation is to be found in a compromise in which we allow a moderate amount of hydration, say 20%, and a corresponding axial ratio of approximately 2.

(18) The Svedberg and K. O. Petersen, "The Ultracentrifuge," Oxford University Press, New York, N. Y., 1940.

The dielectric increment per gram per liter of 0.15 observed for myoglobin is approximately half that of hemoglobin. This difference may arise either from a difference in the nature of the globin in the two cases or might be explained by assuming that hemoglobin is built of four units essentially similar to myoglobin but with a partial stabilization of these units with their electric moments parallel. Any conclusive decision between these alternatives is impossible on the basis of existing data, but the latter must be regarded as the more special one. In any case, in view of the smaller polarization per gram of myoglobin, its greater solubility as compared with hemoglobin should be thought of in terms of its other properties such as lower molecular weight rather than in terms of electric moments.

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Summary

The dielectric properties of a 2.31% aqueous solution of horse myoglobin have been determined over a range of frequency from 1.76 to 15 megacycles. The method employed is based on the use of a cathode ray oscillograph and gives both real and imaginary components of the dielectric constant. The observed value of the static dielectric increment per gram of protein was 0.15 as compared with the value 0.33 for hemoglobin given by Oncley. The critical frequency was found to be 5.5 megacycles. This is approximately twice that of hemoglobin, and half that calculated by Stokes' law, namely, 10.9 megacycles.

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