Weisskopf evaluation of the intensity of a pressure-broadened spectral line applied to the special case of a transition at zero frequency and using the dipole moment matrix element for an inversion transition. The dipole moment of a rotating molecule may be resolved into two components, one perpendicular to the direction of total angular momentum which gives rise to the ordinary rotational spectrum, and one parallel to this direction which can interact with an electromagnetic field only if the molecule undergoes inversion. It is the latter component with which we are concerned.

The Van Vleck–Weisskopf equation specialized as described above becomes

$$\epsilon'' = \frac{4\pi N}{3kT} \Sigma f_{JK} |\mu_{JK}|^2 \frac{\nu\Delta\nu}{\nu^2 + \Delta\nu^2}$$

where N is the number of molecules per cc., f_{JK} is the fractional number of molecules occupying the JK rotational energy level, and $|\mu_{JK}|$ is the dipole moment matrix element for an inversion transition Birnbaum[7,8] gives a closed expression for evaluating the sum

$$<\mu^2_{JK}> = \Sigma f_{JK} |\mu_{JK}|^2$$

in terms of the permanent dipole moment, μ, and the molecular rotation constants, A_0 and B_0. At a given pressure, the maximum loss occurs when $\nu = \Delta\nu$, and at this pressure $\epsilon_0' - \epsilon_\infty' = 2\,\epsilon_M''$.

Table III shows the values of $<\mu^2_{JK}>/\mu^2$ calculated from Birnbaum's expression, values of B_0 as tabulated by Gordy, Smith and Trambarulo[12] and values of A_0 calculated from molecular structural parameters for the gases we have studied. It will be noted that this quantity represents the fraction of the total orientation polarization that is due to the inversion effect. The remaining fraction will disappear as the measuring frequency is increased through the region of rotational absorption for the molecule. Table III also shows the values of $(\epsilon_0' - \epsilon_\infty')$ at a pressure of 100 mm. calculated on the basis of this model and the observed values of $(\epsilon_0' - \epsilon')$. With the exception of the results for CH_3CN, as discussed above, the agreement is quite satisfactory. This lends strong support to the explanation we are proposing for the observed variation of dielectric constant with frequency.

TABLE III

Gas	$<(\mu^2_{JK})>/\mu^2$	$\mu \times 10^{18}$	Theoretical $(\epsilon_0' - \epsilon_\infty') \times 10^5$	Observed $(\epsilon_0' - \epsilon') \times 10^5$
CH_3Cl	0.100	1.87	10.8	10.2
CH_3Br	.073	1.80	8.2	6.6
CH_3CN	.075	3.94	38	49
CHF_3	.415	1.64	37	36
$CHCl_3$	.425	1.01	14	14
CH_3CCl_3	.373	1.79	39	40

Contrary to previous expectations, dielectric dispersion in a gas is not restricted to the region of rotational absorption. A considerable portion of the orientation polarization, depending on the shape of the molecule, disappears at relatively low frequencies due to dispersion associated with low-frequency inversion transitions. Measurements of dielectric constants in the microwave region can be correlated with absorption measurements on the pressure-broadened inversion spectral lines, or the dielectric constant in the microwave region can be calculated accurately from molecular structural parameters.

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(12) W. Gordy, W. V. Smith and R. F. Trambarulo, "Microwave Spectroscopy," John Wiley and Sons, New York, N. Y., 1953.

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA]

Dielectric Properties of Hemoglobin. II. Anomalous Dispersion during Oxygenation

BY SHIRO TAKASHIMA AND RUFUS LUMRY

RECEIVED AUGUST 28, 1957

The dielectric properties of horse and bovine hemoglobin were determined in the frequency range from 50 kc. to 6 mc. as a function of oxygen pressure. The anomalous dispersion which appears in this frequency region is well fitted by the equation of Cole and Cole so that the data can be analyzed in terms of the real component of the dielectric constant ϵ', the mean dielectric relaxation time τ_0 and the distribution parameter for these times α. The dipole moment μ, and τ_0 for unoxygenated horse hemoglobin were 380 debye and 14.5×10^{-8} sec.: for oxygenated horse hemoglobin 430 debye and 10^{-7} sec. and 270 and 12.7×10^{-8} sec. for bovine oxyhemoglobin. In confirmation of previous studies, the difference, $\epsilon_0 - \epsilon_\infty$, in dielectric constant at frequencies below and above the dispersion region, was found to pass through a series of maxima and minima. The maxima occur at about 25 and 75% oxygenation, the central minimum at 50%. Supplementing these findings both τ_0 and α were found to follow the same pattern with the extrema at the same oxygen pressures. α of horse hemoglobin was large at all oxygen pressures, indicating a wide distribution of relaxing species, but that of bovine hemoglobin was much smaller and its change as a function of oxygen was also less pronounced than horse hemoglobin. Aggregation or dissociation of the protein and protein–protein interactions are excluded as sources of phenomena and the possibilities that change in protein shape or charge distribution may be controlled by oxygen pressure are discussed.

In a search for means by which to connect the structure of proteins with their function, hemoglobin quickly presents itself as an interesting subject for experimental attack. Physical and chemical differences between hemoglobin with and without oxygen have been reported.[1] Of most interest among these is the marked difference between the X-ray diffraction patterns and the optical dichroism of the two forms.[2] Indeed the X-ray patterns are so different as to suggest that the internal structure of the molecule becomes drastically altered on oxygen uptake. The significance of this intriguing fact and its possible relationship to the

(1) R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," Interscience Publishers, Inc., New York, N. Y., 1949, Chap. 6.

(2) J. Boyes-Watson, E. Davidson and M. F. Perutz, *Proc. Roy. Soc. (London)*, **A191**, 83 (1947); M. F. Perutz, *ibid.*, **A195**, 474 (1949).

coupling which exists among the four heme groups of the molecule has been discussed by Haurowitz,[3] Wyman and Allen[4] and Keilin.[5] Pauling and coworkers[6] have been led to experiments seeking a change in structure during the addition of inhibitors to hemoglobin and myoglobin. On the basis of the fact that among isocyanides known to be bound equally strongly to isolated heme groups, those with larger size were much less tightly bound to the hemeprotein, they concluded that the heme was buried within the protein in such a way that steric hindrance interferes with the binding reaction. Another method of attack on this problem would be to measure the change in electrical and shape parameters during oxygenation. In the first paper of this series Takashima[7] explored the possibility of dielectric measurement for this problem. Working at a single frequency, 1 megacycle, he observed a sequence of pronounced maxima and minima as oxygen was adsorbed by horse hemoglobin. In order to confirm these findings and to provide more information about the effects and their source, we have applied standard bridge methods to secure the complete dielectric behavior of horse and bovine hemoglobins through the region of anomalous dispersion. The method is particularly powerful in that it provides dipole moments, relaxation times for the dielectric processes involved and a distribution of these times. It is limited in its application by the need to work at very low salt concentrations and limited in its analysis by the uncertainty which presently exists as to the interpretation of dielectric data from protein solutions.

Experimental

Material.—Horse and bovine hemoglobin were purified by the alcohol fractionation method; they were recrystallized three times from water solution. The final alcohol concentrations were 30 and 40% for horse and bovine hemoglobin, respectively. Horse hemoglobin was washed repeatedly with cold conductivity water. It was found that this procedure was sufficient without dialysis to establish the necessary low conductivity. Because of the much higher solubility of bovine hemoglobin, this procedure was not suitable for bovine hemoglobin. Solutions of the latter were dialyzed at low temperature against the conductivity water. Usually the conductivity of horse hemoglobin was so low as to be outside the range of the bridge and so potassium chloride was added to secure a bridge resistance reading of 1000 ohms at 100 kc. The final concentration of salt was about 10^{-5} M and nearly constant from experiment to experiment. The resistance of bovine hemoglobin was usually 800 ohms at 100 kc.

It was necessary to prepare fresh crystalline hemoglobin every one or two weeks. The material does not store well even at low temperatures. The quantitative results of these experiments varied slightly from preparation to preparation and with increasing age of the preparation. Methemoglobin formation appeared to be the principal source of loss in activity.

Procedure.—The solution cell containing hemoglobin solution was evacuated for 20–30 minutes and then filled with a mixture of nitrogen and oxygen. Usually flowing gas was continually passed through the cell. The oxygen partial pressure was determined by standard volumetric techniques or more commonly using a Beckman Oxygen Analyzer Model E-2.[8] The concentration of protein was from 10–15 grams per liter on the basis of dry weights obtained at 110°.

A standard bridge replacement method for measuring impedance was employed throughout. The impedance bridge was a General Radio Model 916 AL covering the frequency range from 50 kc. to 5 mc. The capacity cell was a Teflon cup set into a heavy block of aluminum which was surrounded by coils through which refrigerating oil flowed. A fixed and a movable platinum disk, platinized frequently, served as electrodes. The movable disk was attached to a micrometer head which permitted changes in plate separation to be known to 0.001 in. The cell was gas-tight. Temperature was maintained in the solution within 0.1°. At each frequency resistance and reactance readings at at least two plate separations were made. The equivalent circuit of the cell and solution was determined by trial and error analysis of the bridge values for solutions of salts and small dipolar molecules. It was found possible to calculate conductance and capacitance values using an approximation to the full equation for the admittance of the equivalent circuit which simplified calculations without increasing the over-all error. The over-all procedure fully corrected for electrode polarization in the range of 10^{-5} M salt at the lowest frequencies. At 10^{-4} M KCl further corrections would have been necessary so that most work was carried out in a lower range of conductance. Above 4 mc. the conductance curve for KCl solutions deviates rapidly from the expected straight line. Protein solutions behave in the same way. The effect is thought to be due to uncorrected bridge and cell inductance. Up to 5.5 mc. it was possible to correct for this effect by subtracting from the conductance of the protein that of a KCl solution giving the same low frequency conductance. Above 5.5 mc. the correction was too large to be made with any reliability and the data could not be used. Measurements at frequencies lower than 100 kc. were erratic and it was necessary to average several results to secure a reliable mean value.

As a result of inductance contributions, the high frequency dielectric constant usually could not be determined with any precision experimentally. The values which have been used on subsequent plots were obtained from the formula

$$\epsilon'_\infty = \epsilon'_{H_2O} - (\epsilon'_{H_2O} - 1)\gamma \qquad (1)^9$$

in which ϵ'_∞ is the real dielectric constant for protein solution at high frequency and ϵ'_{H_2O} is the static dielectric constant of water. γ is the fraction of the solution volume which was anhydrous protein.

The low frequency conductance was obtained by plotting conductance *versus* the reciprocal of frequency and extrapolating to low frequencies. The determination of ϵ'' was particularly difficult as is usually the case in experiments of this sort. Errors of 0.5% in the conductance are too large to tolerate. In the worst cases the calculation of ϵ'' was made on the basis of a smooth curve drawn through a number of conductance points rather than for the individual points. The maximum error in ϵ' was about $\pm 0.4\%$.[10]

Most dielectric data and in particular those for polar solutions are well fitted by an empirical expression due to Cole and Cole[11]

$$\epsilon^* = \epsilon'_\infty + \frac{\epsilon'_0 - \epsilon'_\infty}{1 + (i\omega\tau_0)^{1-\alpha}} \qquad (2)$$

in which ϵ^* the complex dielectric constant, is defined by $\epsilon^* = \epsilon' - i\epsilon''$, ϵ'_0 is the dielectric constant below the anomalous dispersion range and ϵ'_∞ that above. τ_0 is the mean relaxation time for whatever may be the dielectric relaxation process, ω is the frequency, and α is the distribution parameter for these relaxation times. α varies from 0 to 1. At $\alpha = 0$, equation 2 reduces to Debye's formula[12]

(3) F. Haurowitz, *Z. physiol. Chem.*, **254**, 266 (1938).

(4) J. Wyman and W. D. Allen, *J. Polymer Sci.*, **7**, 449 (1951).

(5) D. Keilin, *Nature*, **171**, 922 (1953).

(6) R. C. C. St. George and L. Pauling, *Science*, **114**, 629 (1951); A. Lein and L. Pauling, *Proc. Nat. Acad. Sci. U. S.*, **42**, 51 (1956).

(7) S. Takashima, THIS JOURNAL, **78**, 541 (1956).

(8) Beckman Instruments, Inc., Pasadena, California.

(9) According to Shaw, Jansen and Lineweaver this equation gives larger values than the experimental ones. However, the correction is of small magnitude and the error in its use is not significant in this work. T. M. Shaw, E. F. Jansen and H. Lineweaver, *J. Chem. Phys.*, **12**, 439 (1944).

(10) Full details of the experimental procedure and analysis of the data will be supplied by the second author on request.

(11) K. S. Cole and R. H. Cole, *J. Chem. Phys.*, **9**, 341 (1941).

(12) P. Debye, "Polar Molecules," (Chemical Catalog Co.) Reinhold Publ. Corp., New York, N. Y., 1929, Chap. V.

which was originally derived to describe a dispersion process consisting of the rotation of a spherical molecule bearing a fixed dipole. The Cole–Cole equation can be solved explicitly for both τ_0 and α. These are, however, more easily determined from plots suggested by Cole and Cole.[13] Debye's expressions for the real and imaginary parts of the dielectric constant, in which α is zero, can be combined to give

$$\left(\epsilon' - \frac{\epsilon'_0 - \epsilon'_\infty}{2}\right)^2 + \epsilon''^2 = \left(\frac{\epsilon'_0 - \epsilon'_\infty}{2}\right)^2 \quad (3)$$

Hence ϵ'' graphed against ϵ' as abscissa gives a complete semi-circle above the real axis of the imaginary plane with diameter $\epsilon'_0 - \epsilon_\infty$ and center on the abscissa. If, however, α is not zero, as is usually the case, a semi-circle still results but the center of its circle lies below the real axis. The real axis and a radius drawn from the center to either point at which the arc cuts that axis makes an angle $\alpha(\pi/2)$ (see Fig. 1). The distance between the two points of intersection with the real axis is still $\epsilon'_0 - \epsilon'_\infty$. The maximum value of ϵ'' occurs at the critical frequency f_c which is related to τ_0 according to $\tau_0 = 1/2\pi f_c$. The values of the three parameters are thus determined from Cole–Cole plots. f_c can also be measured from plots of ϵ' *versus* logarithm frequency and a comparison of the two values provides a good check on the applicability of the method of analysis and on the goodness of curve fitting. Good agreement was usually found and served as a criterion for acceptance of data.

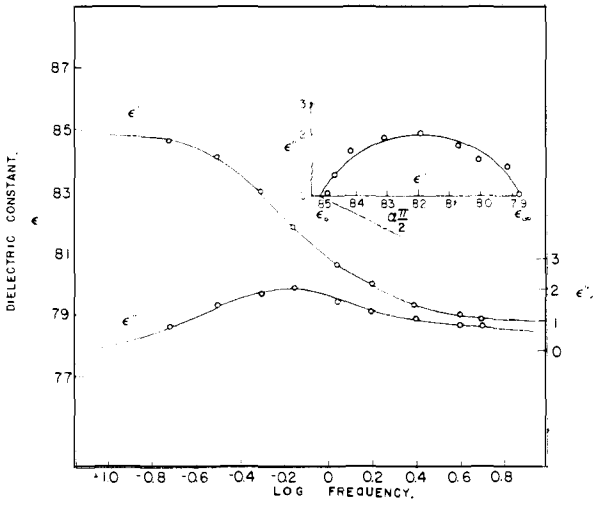

Fig. 1.—The variation of the real, ϵ', and the imaginary, ϵ'', components of the dielectric constant of oxyhemoglobin through the anomalous dispersion range. The insert is the Cole–Cole plot of the same data.

The dipole moment μ can be estimated from the expression given by Oncley[14]

$$\mu^2 = (9000kT/4\pi Nh)\left(M\frac{\epsilon'_0 - \epsilon'_\infty}{g}\right) \quad (4)$$

in which k is the Boltzmann constant, N, Avogadro's number, M, the molecular weight, g, grams per liter of protein, and h is an empirical parameter which has been taken as 8.5 after Wyman.[15]

Results

Horse Hemoglobin.—Typical plots of the behavior of ϵ' and ϵ'' *versus* logarithm frequency and the corresponding Cole–Cole plot are given in Fig. 1 which was determined with horse oxyhemoglobin. The maximum of ϵ'' should occur at the same frequency as the mid-point of the ϵ' curve as it does. Within the experimental error the Cole–Cole plot is an arc of a circle and its mid-point lies below the ϵ' axis. α is thus not zero for oxyhemoglobin at this temperature nor was it found to be so at any stage of oxygenation. The total dielectric increment, relaxation time and Cole–Cole parameter for horse oxyhemoglobin from Fig. 1 were 0.59, 10.1 × 10^{-8} sec. and 0.3, respectively. These quantities vary slightly from preparation to preparation.

In Fig. 2 are shown the values of dielectric increment, $\delta\epsilon'/g$, and τ_0 for horse hemoglobin at 15° as a function of the partial pressure of oxygen. In these experiments the conductance varied from 900 to 1300 mhos and the concentration of protein was 13 to 15 g./l. The solution was equilibrated with the gas mixture for 2 to 3 hours as required to achieve equilibrium. At the extreme right of the figure the values for the solution in equilibrium with air are given, and it will be noticed that they are the same as those at 9 mm. Other studies at oxygen pressures greater than that necessary to saturate the protein demonstrated that no further changes occurred once the protein became saturated. Experiments on other samples of hemoglobin were in semi-quantitative agreement with that of Fig. 2. Although the values of the calculated quantities varied slightly, the maxima and minimum were at the same oxygen pressures.

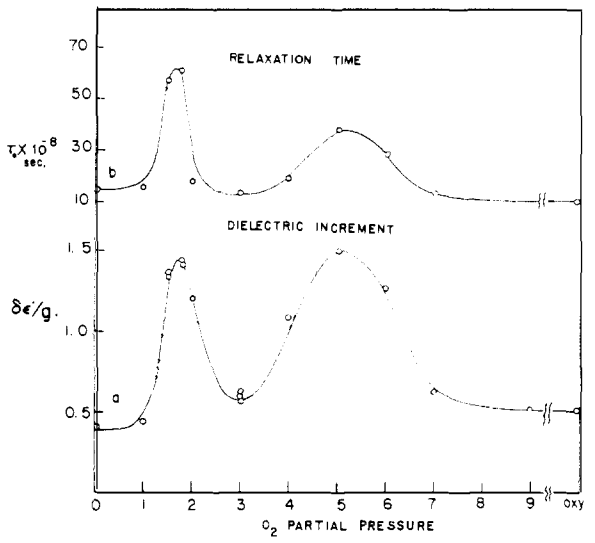

Fig. 2.—$(\epsilon_0' - \epsilon_\infty')/g$ and the relaxation time as a function of oxygen partial pressure. The points following the break are for air at one atmosphere. Pressures are in mm.

The dielectric increment results are very similar to those reported for horse hemoglobin in the first paper of this series[6] in which the increment was shown to pass through a series of minima and maxima. The oxygen pressures at which the extrema occur were not all the same. In Fig. 2 the maxima are shown to lie at 1.7 and 5 mm. and the central minimum is at 3 mm. In the original work the maxima were found at 1 and 6 mm. the position of the minimum being the same within error.

The relaxation times were found to pass through a similar series of changes with extrema at the same oxygen pressures (Fig. 2). So also did the Cole–Cole parameter as shown in Fig. 3. The dipole moments and numerical values for the empirical

(13) C. P. Smyth, "Dielectric Behavior and Structure," McGraw-Hill Book Co., Inc., New York, N. Y., 1955, Chap. 11.

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

(15) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, Chap. 22.

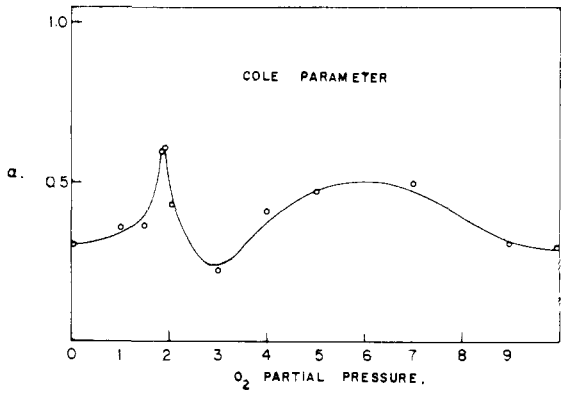

Fig. 3.—The variation in the Cole–Cole distribution parameter, α, as a function of oxygen partial pressure. Pressures are in mm.

dielectric quantities are presented in Table I. The Cole–Cole plots at the oxygen pressures of the extrema are given in Fig. 4 together with the frequencies in megacycles.

Special mention should be made of the results at 1.7 mm. Because of the large value of α, the ϵ' *versus* logarithm frequency plot was very flat. As a consequence there was considerable arbitrariness in evaluating both the low frequency value of ϵ' and of τ_0. The dielectric quantities listed in Table I for this pressure are based on a rather large extrapolation of the Cole–Cole plot to frequencies beyond our bridge and are certain to be in larger error than the other points.

In confirmation of earlier findings[6] the dielectric increment was found to be a constant function of protein concentration in the range from 3 to 15 g./l., the highest measured. If hemoglobin is not extremely fresh, there are deviations below 3 g./l.

TABLE I

THE DIELECTRIC INCREMENT, DIPOLE MOMENT, RELAXATION TIME AND THE COLE–COLE PARAMETER OF HEMOGLOBIN AT INTERMEDIATE STATES OF OXYGENATION

	O_2 part. press (mm.)	$(\epsilon'_0 - \epsilon'_\infty)/g$	μ(debye)	$\tau_0 \times 10^{-8}$, sec.	α
Horse	Reduced	0.40	380	14.5	0.302
	1.0	0.43	380	15.2	.365
	1.5	1.37	710	56.9	.362
	1.7	1.44	720	60.9	.600
	2.0	1.20	670	17.9	.422
	3.0	0.60	470	14.1	.223
	4.0	1.09	630	17.1	.415
	5.0	1.50	740	37.4	.475
	6.0	1.27	680	28.4	...
	7.0	0.60	470	12.9	.500
	9.0	.50	430	..	.313
	Oxy	.50	430	10.1	.295
Bovine	1.0	.26	310	10.5	.26
	3.0	.38	370	15.9	.49
	6.0	.18	260	10.5	.06
	11.0	.35	340	15.9	.53
	Oxy	.21	270	12.7	.08

Bovine Hemoglobin.—For fully oxygenated bovine hemoglobin, the dielectric increment is 0.26 per gram and thus considerably smaller than the value of 0.5 for horse hemoglobin. Also all the

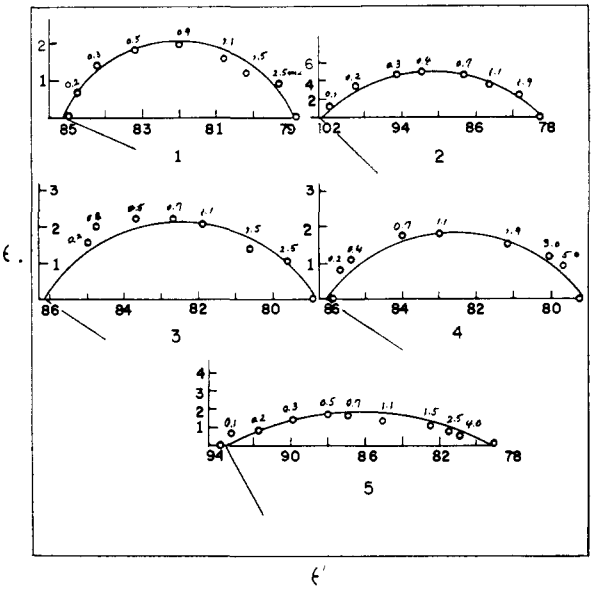

Fig. 4.—Cole–Cole plots of the data obtained at several oxygen pressures: (1) oxyHb; (2) 5 mm.; (3) 3 mm.; (4) RedHb; (5) 1.7 mm. The numbers on these figures are the experimental frequencies in megacycles.

other parameters are quantitatively different for two kinds of hemoglobin. The most extreme difference between the oxyhemoglobins to be noticed in Table I is in the Cole–Cole parameter. Horse oxyhemoglobin gave a large value of α, indicative of a wide distribution of relaxation times. The small α for bovine oxyhemoglobin suggests a much more homogeneous protein which can very nearly be characterized by a single relaxation time.

The dielectric parameters were determined as a function of oxygen partial pressure. Figure 5 gives the dielectric increment and the relaxation time at several oxygen pressures. The qualitative pattern of change in $\delta\epsilon'/g$ and τ_0 is identical with that reported for horse hemoglobin. Quantitative differences occur in the position of the peaks and in the oxygen pressure range in which the effects occur. The two maxima in both plots of Fig. 5 appear at 3 and 11 mm. The minimum is at 6 mm. in both. The maximum to minimum values and the maxima themselves are considerably smaller than observed with horse hemoglobin. For example, the highest value of the dielectric increment measured with bovine hemoglobin was 0.38 as compared with 1.50 for horse hemoglobin.

The distribution parameter α for bovine hemoglobin also follows the standard pattern with extrema at the same oxygen pressures as the other dielectric quantities (Fig. 6). α is very large at the peaks of the other parameters and very small at the minima. Numerical values for the extrema are also given in Table I. Only α shows variations of the same size as observed with horse hemoglobin.

The small value of α at the central minimum indicates a very small spread of relaxation times. This is surprising since the central minimum occurs at an oxygen pressure at which several intermediate stages of oxygenated hemoglobin exist simultaneously.

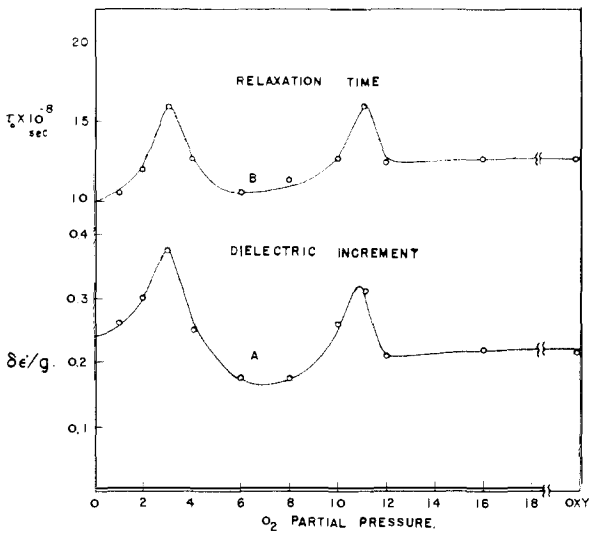

Fig. 5.—The behavior of the dielectric increment (curve A) and the relaxation time (curve B) for bovine hemoglobin solution as a function of oxygen pressure at 15°. Pressures are in mm.

The dielectric behavior of solution of KCl and ovalbumin were investigated as a function of oxygen pressure to establish in a limited way that oxygen itself or some non-specific effect of oxygen on proteins was not the source of the unusual behavior of hemoglobin. Oxygen addition was found to be completely without effect in these cases.

Since the solutions were unbuffered, the *p*H was that of the nearly isoionic protein and undoubtedly varied slightly with oxygen pressure as a result of the Bohr effect.

Discussion

Horse Hemoglobin.—There is no generally satisfactory theory for the quantitative dielectric behavior of polar liquids. Cole–Cole plotting of the data for a number of types of polar solutions has demonstrated that the Cole–Cole equation provides a highly precise fit for the data. It is not, however, unique nor does it rest on complete theoretical arguments. Shaw and co-workers[11] using a bridge of higher precision than that employed here reported extensive results with β-lactoglobulin which conform closely to Cole–Cole plots. This is the only report of data of sufficient precision to test the applicability of this method of analysis for proteins. As can be judged from Figs. 1 and 4, the data of this paper are satisfactorily fitted in this way,[16] and it will be noted that the calculated high frequency points (those at low ϵ') lie on the curves.

The data of Figs. 2 and 3 and Table I are the most complete and well-controlled set which were obtained. There were however extensive measurements on other preparations and even with another impedance bridge all in semi-quantitative agreement with the reported results. In no case was there a deviation from the basic pattern of maxima

(16) More extensive tests of the fit to Cole–Cole plots were carried out at 25° using carbon monoxide instead of oxygen. The agreement was remarkably good; S. Takashima and R. Lumry, THIS JOURNAL, **80**, 4244 (1958).

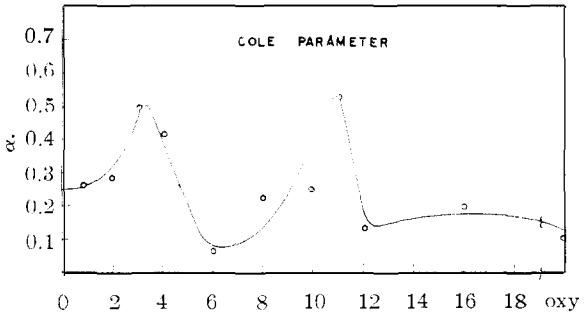

Fig. 6.—The Cole–Cole distribution parameter α as a function of oxygen pressure at 15°. Pressures are in mm.

and minima. Confirmation of Takashima's original observation[4] on the real dielectric constant is thus complete. The small quantitative differences which have appeared can be attributed to the variability of the experimental material and the fact that the original experiments were carried out at a single frequency. The quantitative fluctuations from experiment to experiment and the approximations in the present calculations do not cast any doubt on the generality and reproducibility of the major pattern of changes in any of the three parameters, $\delta\epsilon'/g$, τ_0 and α. Although α is a sensitive function of conductance, the quantity least precisely measured in these experiments, so that the quantitative significance of Fig. 3 is in some doubt, the general pattern is most certainly present in this parameter as in the others.

Correlation of the extent of oxygen saturation at a given oxygen pressure with the dielectric changes is complicated by the considerable sensitivity of the oxygenation equilibria to ionic strength.[17] However, the data available for horse hemoglobin cover the range of ionic strengths of these experiments. In unbuffered solutions and in those buffered with 10^{-4} phosphate, saturation occurs at about 12 or 13 mm. of oxygen. Figures 4 and 6 are seen to become flat at 9 or 10 mm. Thus though there is a rough agreement between the ranges of pressures in the two types of experiments, it is not as exact as could be desired. The first and second maxima occur at about 25 and 75% saturation. The central minimum is at 50%. Further increases in oxygen pressure beyond 10 mm. produce no further changes.

The oxygenation of hemoglobin is thought to involve a series of one, two, three and four oxygen-molecule additions. The quantitative aspects of these equilibria have not been determined at sufficiently low ionic strengths to be useful for the comparison with our data. On the basis of present data it does not seem possible to reconcile the various dielectric changes with the several intermediate states of oxygenation. In the first place unless the relative magnitudes of the equilibrium constants are greatly changed at low ionic strength, there are always appreciable populations of the intermediate oxygen compounds at all pressures except those near zero and those near saturation. If, for example, we attempt to identify the first maximum

(17) S. Takashima, *ibid.*, **77**, 6173 (1955).

with the form HbO_2 and the second with the form HbO_6, there should not be a central minimum in the curves but rather a higher maximum at that point, for these two species reach their maximum concentration in the mid-region of pressures. Similarly the high values of the dipole moment at the maxima represent averages for the several species present at these points. If these averages are corrected to secure the dipole moment of pure HbO_2 or HbO_6, the values are very much larger than anything thus far measured for a pure protein. The lack of agreement between the results of the two kinds of experiments thus presents a troublesome problem which may however be solved by a study of the oxygenation equilibria at low salt concentrations.

Bovine Hemoglobin.—Bovine hemoglobin demonstrates on oxygenation the same dielectric behavior as horse hemoglobin. This conclusion is established in Figs. 5 and 6, where it is shown that the dielectric increment (and hence the dipole moment), the mean relaxation time and the distribution parameter follow an identical series of changes. The extrema for each parameter occur at the same set of oxygen pressures, within our errors. Quantitative differences between horse and bovine hemoglobin occur not only in the ordinate values in these figures but also in the positions of the minima and maxima and the total range of pressures over which the effects occur. The latter difference may be correlated with the reduced affinity of bovine hemoglobin for oxygen, which will extend the effects to higher pressures. Hill and Wolvekamp studied the oxygenation of bovine hemoglobin at 25° in buffered solutions of high ionic strength.[18] Since the oxygenation of hemoglobin is sensitive to ionic strength,[17] a comparison of the points of their isotherm with our results secured at low ionic strength is not strictly applicable. However, the dielectric increment was roughly determined at 25° as a function of oxygen pressure and is given in Table II along with the data of Hill and Wolvekamp. The agreement in oxygen range for the two kinds of experiments is good considering the differences in ionic strength. It will also be noticed by comparing these values with those at 15° that the increase in temperature shifts the whole curve toward higher oxygen pressures.

TABLE II

THE COMPARISON OF SPECTROSCOPIC AND DIELECTRIC EXPERIMENTS ON OXYGENATION OF BOVINE HEMOGLOBIN

Spectroscopic		Dielectric	
Degree of satn.	Oxygen press.		Oxygen pressure
25	6.0	First max.	4.2
50	11.3	Minimum	9.0
75	14.8	Second max.	13.3
100	20	No further change above	18–20

There are several possible explanations for the change in mean relaxation time with oxygenation: (1) changes in the size of the hemoglobin molecule, swelling or shrinking, (2) changes in the state of aggregation, (3) changes in the distribution of charge, (4) changes in the shape of the protein, (5) changes in the intermolecular interaction between protein molecules and (6) changes in the electrostatic interaction between protein and solvent. Not all of these are, of course, independent.

Changes in size are simply related to rotational relaxation times and could be brought about by variation in the amount of internally sequestered water or in that held in the external hydration shell. Application of the Stokes equation relating rotational relaxation times for a sphere with its radius requires that the radius change by a factor of two between first maximum and central minimum. While this appears to be an unreasonably large change, present data do not eliminate it as a possibility.

Changes in aggregation are not consistent with the observation that the dielectric increment is independent of concentration. One of us determined this relationship over the entire oxygen range and found it constant up to concentrations well outside the present experimental range.[19] This fact eliminates aggregation as a possibility and also dissociation, for if hemoglobin dissociates into two identical subunits, as is thought,[1] the position of the dissociation equilibrium would also be determined by the total concentration of protein. An explanation based on interprotein interactions is also eliminated by the independence of dielectric properties on protein concentration.

Changes in shape during oxygenation could be consistent with the X-ray diffraction evidence. However, Nakamura found no change in the viscosity of horse hemoglobin solutions over a wide range of oxygen pressures.[20] Unfortunately these experiments were carried out in 0.2 *M* phosphate buffer at 25°. Both temperature and salt concentration were unfavorable. The dielectric effects are much less pronounced at 25°.[20] Also high salt may well wash out the dielectric effects just as it eliminates the coupling between heme groups.[17] Further viscosity studies at low ionic strengths will be necessary to establish the absence of changes of shape.

It seems probable that the dielectric effects must be related to the internal structural rearrangements indicated by X-ray and optical studies but such changes will not necessarily produce detectable alterations in shape. In this connection it is interesting to note that Klotz and Heiney[21] have reported large shifts in optical rotation during the oxygenation of the respiratory protein hemocyanin. Such changes are not conclusively interpreted in terms of internal structural alteration because of the visible absorption spectrum of this non-porphyrin protein but they are certainly suggestive that such alterations do occur.

Evidence for or against an altered interaction with the solvent is wholly lacking. Similarly there

(18) R. Hill and H. P. Wolvekamp, *Proc. Roy. Soc.* (*London*), **B120**, 484 (1936).

(19) S. Takashima, unpublished observations.

(20) T. Nakamura, *J. Biochem.* (*Japan*), **42**, 6 (1955). Our experiment at 25° revealed that the changes of dielectric increment were less pronounced and only a very small change was observed in the relaxation time during the oxygenation, which seems to be consistent with Nakamura's observation; S. Takashima and R. Lumry, unpublished observations.

(21) I. Klotz and R. E. Heiney, *Proc. Nat. Acad. Sci. U. S.*, **43**, 717 (1957).

is no pertinent evidence relating directly to changes in charge distribution. The alterations in dipole moment which take place could be entirely the result of a change in shape. On the other hand there is increasing evidence for unusual interaction of charges in proteins which may be sensitive to the physiological state of the protein. The classical picture of Debye in which a rigid sphere of fixed dipole moment rotates in solution no longer appears entirely satisfactory. Such a molecular picture is particularly inconsistent with Jacobsen's dielectric studies[22] on folded poly acid–base macromolecules. When these molecules in solution were fully oriented in an annulus across which there was a high shear gradient, the dielectric properties were the same whether measured across the long axis or across the short axis of the molecules. He also made comparisons between the dielectric relaxation time and rotational relaxation times obtained in other ways and found poor agreement. As a substitute for the rotational picture of Debye, he suggested that proteins have a large effect in organizing the structure of water and that it is this structure as modified by the protein which is seen in dielectric experiments. Recently Kirkwood and Shumaker[23] have proposed an entirely new molecular picture for proteins in which the response of the permanent dipole moment, if any, to an applied field is equalled or overwhelmed by proton fluctuations following the field. Since there are more basic sites in proteins than bound protons, the protons may migrate from site to site toward the end of the protein at which the negative field intensity is the greatest. This is somewhat similar to the Maxwell–Wagner theory in which the ionic atmosphere surrounding a polyelectrolyte migrates in an applied field. Another application of the Maxwell–Wagner theory to a polyelectrolyte recently has been made by Dintzis, *et al.*[23,24]

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

(23) J. G. Kirkwood and J. B. Shumaker, *Proc. Nat. Acad. U. S.*, **38**, 855 (1952).

(24) H. M. Dintzis, J. L. Oncley and R. M. Fuoss, *ibid.*, **40**, 62 (1954).

Since the fluctuations of charge should also result in interprotein interactions, it has been possible to seek some verification of the Kirkwood–Shumaker theory from studies of these effects. Timasheff and co-workers[25] report some experimental confirmation of charge fluctuation as a source of protein interaction from light-scattering studies. It seems possible that a change in protein charge distribution on oxygenation might influence the charge fluctuation phenomenon and thus appear as a change in effective dipole moment. On the other hand the relaxation process in this theory seems to be attributable to the formation or dispersion of the hydration shells of the charges as they move about, and it is not at all clear how a change in charge distribution would alter the relaxation time. Unfortunately the testing of the various theories and the application of the newer ones to dielectric phenomena are not simple nor well advanced. It is thus not possible at present to interpret the behavior of hemoglobin in terms of a well-established picture of molecular events. In any event the experimental results reported here appear to be reproducible and free of artifacts. Furthermore at least two kinds of hemoglobin demonstrate the same behavior and thus serve to suggest a generality for the phenomenon under observation. It thus appears for at least one protein that physiological function is associated with drastic modifications in the properties of the solutions of that protein.

Acknowledgments.—This research was supported by the United States Navy through the Office of Naval Research under contract Nonr 710(15) NR 120-306. Additional equipment was supplied by the Graduate School, University of Minnesota. We are indebted to Professors O. Schmitt and H. Schwan for their very helpful assistance.

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

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Dielectric Properties of Hemoglobin. III. Carbon Monoxide Addition to Horse Hemoglobin

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RECEIVED AUGUST 28, 1957

The dielectric parameters have been determined for horse hemoglobin through the frequency region of anomalous dispersion as a function of the degree of saturation with carbon monoxide. The dipole moment of carboxyhemoglobin was 410 debye in excellent agreement with Oncley's value, but the mean relaxation time τ_0 was 10^{-7} sec., 15% larger than reported by Oncley. The real and imaginary parts of the dielectric constant plotted against each other formed good semi-circles in agreement with the equation of Cole and Cole. As carbon monoxide was added, the dielectric quantities showed the pattern of maxima and minima previously reported from oxygenation studies. The pattern was clearly marked in all parameters at 15° but only detectable in τ_0 at 25° and not in the dielectric increment or distribution parameter for relaxation time α. At 15° there are maxima at about 35 and 75% saturation and a minimum at about 55%. The maximum to minimum differences are considerably smaller than observed in oxygenation experiments. The distribution of relaxation times is broad at 15° and narrow at 25°. The difference in τ_0 at the two temperatures is not consistent with the difference in solvent viscosity.

It is well known that the reactions of hemoglobin with carbon monoxide are qualitatively very similar to those with oxygen.[1] In previous papers we already have presented evidence that large changes of unknown molecular nature occur during the oxygenation of hemoglobin.[2,3] These changes pro-

(1) R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," Interscience Publishers, Inc., New York, N. Y., 1949.

(2) S. Takashima, THIS JOURNAL, **78**, 541 (1956).

(3) S. Takashima and R. Lumry, *ibid.*, **80**, 4238 (1958).