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MICROWAVE X-BAND FARADAY ROTATION OF HEMOGLOBIN
WITH ADSORBED WATER

by

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ABSTRACT

The structure of bound water molecules in crystalline, bovine hemoglobin powder is investigated with a microwave technique of Faraday rotation at a frequency of 9.36 Gc. An expression for the Faraday rotation due to displacement current Hall field is derived in terms of dipole concentrations of free water molecules and is applied to measure the changes from free water to bound water molecules on hemoglobin with variable amounts of adsorbed water. In agreement with the theory, experimental result shows a linear relationship between the angle of rotation and the concentration of free water dipoles up to the hydration level of 0.13 gram of water per gram of dry hemoglobin. Above this hydration level, the formation of ice-like structures on the surface of hemoglobin is discussed based on our experimental results.

I. INTRODUCTION

The influence of bound water around proteins has been investigated by measuring the dc conductivity¹ and the dielectric constant^{2,3} of hydrated proteins as a function of adsorbed water. Both the dielectric constant and the conductivity increase as the amount of adsorbed water increases. These observations were interpreted as showing the strong dependence of current on the amount of adsorbed water which changes the dielectric constant of the protein. The increasing dielectric constant of protein with increasing water content was regarded as responsible for the formation of a hydration layer on the surface of the protein. Water of hydration was believed to have an "ice-like" structure since ice forms the most stable hydration layer and has a larger dielectric constant than water. However, it seems necessary to present an experimental confirmation of the formation of an ice-like structure on the surface of the hydrated protein.

Since, at x-band frequencies, dipoles of water molecules are free to rotate in water structure but no longer free to rotate in ice structure,⁴ and in addition, microwave techniques eliminate the tedious problems associated with the manufacture of ohmic contacts, a greatly

improved microwave technique of Faraday rotation was developed. This technique was applied in order to observe the effect of dipole concentration of the water molecules as a function of the hydration of crystalline hemoglobin powder samples.

II. FARADAY ROTATION

The plane of a linearly polarized electromagnetic wave, propagating through a sample medium with dc magnetic field in the direction of propagation, is rotated according to the theory of Faraday rotation.^{5, 6} Depending on the physical conditions, some authors have used several closely related but nevertheless different mechanisms to explain Faraday rotation. In the case of organic or biological substances such as proteins, it is very hard to choose an appropriate model for analysis because many physical parameters are not yet known. This problem is simplified by treating the medium as having the electrical properties of conductivity σ , permeability μ , and permittivity ϵ .

A. Hall Effect and Faraday Rotation

Faraday rotation based on Hall effect has been analyzed by Barlow⁶ and reviewed by Engineer and Nag.⁷ Barlow assumed that both conduction (mobile charge) and displacement (oscillating dipoles) currents in the medium interact with an applied magnetic field to generate Hall fields. Engineer and Nag formulated a form of Maxwell's equations, which included the Hall field, in such a manner that they obtain results consistent with the theory of free-carrier motion.⁵

Using mks units, Maxwell's equations, including the Hall field,
can be written as:

$$\underline{\nabla} \times \underline{E} = -\mu \frac{\partial \underline{H}}{\partial t} \quad (1)$$

$$\underline{\nabla} \times \underline{H} = \underline{J}_{\text{total}} = \underline{J}_C + \underline{J}_D,$$

where $\underline{J}_C = \sigma (\underline{E} + \underline{E}_{HC})$ (2)

$$\underline{E}_{HC} = R_C \underline{B} \times \sigma (\underline{E} + \underline{E}_{HC})$$

and $\underline{J}_D = \epsilon_0 \frac{\partial \underline{E}}{\partial t} + (\epsilon - \epsilon_0) \frac{\partial}{\partial t} (\underline{E} + \underline{E}_{HD})$ (3)

$$\underline{E}_{HD} = R_D \underline{B} \times (\epsilon - \epsilon_0) \frac{\partial}{\partial t} (\underline{E} + \underline{E}_{HD}),$$

where

- \underline{J}_C : conduction current density,
- \underline{J}_D : displacement current density,
- \underline{E}_{HC} : Hall field due to conduction current,
- \underline{E}_{HD} : Hall field due to displacement current,
- R_C : Hall constant for conduction current,
- R_D : Hall constant for displacement current,
- \underline{B} : dc magnetic field,
- σ : conductivity of medium,
- ϵ : permittivity of medium,
- ϵ_0 : permittivity of free space.

We choose a system of axes such that the direction of propagation of the TE mode wave is along the z-direction which is parallel to the applied static magnetic field B. If we resolve a linearly polarized wave of angular frequency ω incident upon the medium into two, counter-rotating, circularly polarized waves, propagation constants K_+ and K_- for the left and righthand circularly polarized waves can be calculated as,

$$K_{\pm}^2 = \omega^2 \mu \epsilon_0 \left\{ \frac{\pm \omega (\epsilon - \epsilon_0) R_D B - [\omega (\epsilon - \epsilon_0) R_D B]^2}{1 - [\omega (\epsilon - \epsilon_0) R_D B]^2} \right\} + \omega^2 \mu \epsilon \left\{ \frac{1 \mp \omega (\epsilon - \epsilon_0) R_D B}{1 - [\omega (\epsilon - \epsilon_0) R_D B]^2} \right\} - j \omega \mu \sigma \left\{ \frac{1 \pm j \sigma R_C B}{1 + (\sigma R_C B)^2} \right\}. \quad (4)$$

In the limit of small B, we can neglect the squared terms in Eq. 4 by expressing

$$\sigma R_C B = \frac{E_{HC}}{E + E_{HC}} < 1 \quad (E > E_{HC}) \quad (5)$$

$$\omega (\epsilon - \epsilon_0) R_D B = \frac{E_{HD}}{E + E_{HD}} < 1 \quad (E > E_{HD})$$

Under these conditions, Eq. 4 reduces to⁷

$$K_{\pm}^2 = -j \omega \mu (\sigma + j \omega \epsilon) \pm \omega \mu \left[\sigma^2 R_C B - \omega^2 (\epsilon - \epsilon_0)^2 R_D B \right]. \quad (6)$$

B. The Angle of Faraday Rotation

The propagation constants of the circularly polarized waves are expressed in terms of attenuation constant, α_{\pm} , and phase constants, β_{\pm} , by

$$jK_{\pm} = \alpha_{\pm} + j\beta_{\pm}.$$

Since β_+ and β_- are not the same, thus implying that two circularly polarized waves travel with different phase velocities, then as the resulting linearly polarized wave emerges from the sample medium of thickness s it will be rotated by an angle θ given by

$$\theta = \frac{1}{2} (\beta_- - \beta_+) s. \quad (7)$$

The emerging wave is also slightly elliptically polarized due to $\alpha_+ \neq \alpha_-$.

Expressing Eq. 6 in the form

$$(jK_{\pm})^2 = A \mp B,$$

with $A \equiv j\omega\mu(\sigma + j\omega\epsilon),$

$$B \equiv \omega\mu \left[\sigma^2 R_C B - \omega^2 (\epsilon - \epsilon_0)^2 R_D B \right],$$

we can write

$$(jK_-) - (jK_+) = (\alpha_- - \alpha_+) + j(\beta_- - \beta_+) = \sqrt{A+B} - \sqrt{A-B} \cong \frac{B\sqrt{A}}{A}$$

by noting the condition $|A| > |B|$ so that

$$\beta_- - \beta_+ = \text{Imaginary component of } \frac{B\sqrt{A}}{A} \quad (8)$$

We now consider the \sqrt{A} by defining

$$\sqrt{A} = \alpha_0 + j\beta_0$$

and consequently

$$\alpha_0 = \omega \left(\frac{\mu\epsilon}{2} \right)^{\frac{1}{2}} \left[\left(1 + \frac{\sigma^2}{\omega^2 \epsilon} \right)^{\frac{1}{2}} - 1 \right]^{\frac{1}{2}},$$

$$\beta_0 = \omega \left(\frac{\mu\epsilon}{2} \right)^{\frac{1}{2}} \left[\left(1 + \frac{\sigma^2}{\omega^2 \epsilon} \right)^{\frac{1}{2}} + 1 \right]^{\frac{1}{2}}.$$

Then the angle of rotation is derived from Eqs. 7 and 8 as,

$$\theta = \frac{s}{2} \frac{\left[\omega^2 (\epsilon - \epsilon_0)^2 R_D B - \sigma^2 R_C B \right] \left[\alpha_0 \sigma + \omega \epsilon \beta_0 \right]}{\sigma^2 + (\omega \epsilon)^2} \quad (9)$$

In the limit of small losses in the sample, $\omega \epsilon \gg \sigma$, which is the case of proteins at microwave frequencies,

$$\alpha_0 \cong 0,$$

$$\beta_0 \cong \omega (\mu\epsilon)^{\frac{1}{2}}.$$

Substituting these values into the expression of the angle in Eq. 9, and considering $\mu = \mu_0$ (air permeability), we obtain

$$\theta = \frac{1}{2} B \cdot S (\epsilon_r)^{-\frac{1}{2}} \left(\frac{\mu_0}{\epsilon_0} \right)^{\frac{1}{2}} \left[\omega^2 (\epsilon - \epsilon_0)^2 R_D - \sigma^2 R_C \right] , \quad (10)$$

where ϵ_r is the relative permittivity, i. e., the ratio of material permittivity to that of air.

Equation 10 can be further approximated for a sample in which the displacement current is large in comparison with the conduction current. This is the case with a powder sample of proteins. When $\omega^2 (\epsilon - \epsilon_0)^2 R_D \gg \sigma^2 R_C$, Eq. 10 reduces to

$$\theta = \frac{1}{2} B \cdot S (\epsilon_r)^{-\frac{1}{2}} \left(\frac{\mu_0}{\epsilon_0} \right)^{\frac{1}{2}} \omega^2 (\epsilon - \epsilon_0)^2 R_D . \quad (11)$$

III. INTERPRETATION OF THE ANGLE FOR HEMOGLOBIN WITH ADSORBED WATER

In order to interpret the measured angles of rotation as a function of the amount of adsorbed water in hemoglobin, we must generalize the system by assuming that the total current density is due to both the hemoglobin itself and the adsorbed water molecules. By simply defining $\Delta\sigma$ and $\Delta\epsilon$ as the additional contribution of conductivity and dielectric constant of adsorbed water molecules to the hydrated hemoglobin, we can write the general system as

$$\begin{aligned} \underline{J}_C &= \sigma \underline{E} = (\sigma^{\text{Hb}} + \Delta\sigma) \underline{E}, \\ \underline{J}_D &= \epsilon \frac{\partial \underline{E}}{\partial t} = (\epsilon^{\text{Hb}} + \Delta\epsilon) \frac{\partial \underline{E}}{\partial t}, \end{aligned} \tag{12}$$

where σ^{Hb} and ϵ^{Hb} represent the conductivity and dielectric constant of hemoglobin, respectively, and $\Delta\sigma$ and $\Delta\epsilon$ are, respectively, the incremental values of conductivity and dielectric constant due to the adsorbed water molecules.

In the presence of a magnetic field, Eq. 12 can be written as:

$$\underline{J}_C = \sigma^{\text{Hb}} (\underline{E} + \underline{E}_{\text{HC}}^{\text{Hb}}) + \Delta\sigma (\underline{E} + \underline{E}_{\text{HC}}^{\text{W}}), \tag{13a}$$

where

$$\underline{E}_{HC}^{Hb} = R_C^{Hb} \underline{B} \times \sigma^{Hb} (\underline{E} + \underline{E}_{HC}^{Hb}), \quad (13b)$$

$$\underline{E}_{HC}^W = R_C^W \underline{B} \times \Delta\sigma (\underline{E} + \underline{E}_{HC}^W), \quad (13c)$$

and

$$\begin{aligned} \underline{J}_D = & \left[\epsilon_0 \frac{\partial \underline{E}}{\partial t} + (\epsilon^{Hb} - \epsilon_0) \frac{\partial}{\partial t} (\underline{E} + \underline{E}_{HD}^{Hb}) \right] \\ & + \left[\epsilon_0 \frac{\partial \underline{E}}{\partial t} + (\Delta\epsilon - \epsilon_0) \frac{\partial}{\partial t} (\underline{E} + \underline{E}_{HD}^W) \right], \end{aligned} \quad (14a)$$

where

$$\underline{E}_{HD}^{Hb} = R_D^{Hb} \underline{B} \times (\epsilon^{Hb} - \epsilon_0) \frac{\partial}{\partial t} (\underline{E} + \underline{E}_{HD}^{Hb}), \quad (14b)$$

$$\underline{E}_{HD}^W = R_D^W \underline{B} \times (\Delta\epsilon - \epsilon_0) \frac{\partial}{\partial t} (\underline{E} + \underline{E}_{HD}^W), \quad (14c)$$

where R_C^{Hb} and R_C^W are Hall constants for the conduction currents of hemoglobin and water, respectively. Similarly, R_D^{Hb} and R_D^W are Hall constants for the displacement currents of hemoglobin and water. The first term of Eq. 14a represents the displacement current due to the hemoglobin itself; the second term represents the displacement current due to the adsorbed water molecules in the presence of Hall fields.

Proceeding as in the previous derivation for the case of only one displacement current, we arrive at an equation for θ as the sum of two

terms

$$\theta = \theta_{\text{Hb}} + \Delta\theta_{\text{W}} . \quad (15)$$

Since both hemoglobin and adsorbed water have the displacement currents larger than their conduction currents, we can obtain the solution of θ_{Hb} and $\Delta\theta_{\text{W}}$ by inspection:

$$\theta_{\text{Hb}} = \frac{1}{2} B \cdot S (\epsilon_{\text{r}}^{\text{Hb}})^{-\frac{1}{2}} \left(\frac{\mu_0}{\epsilon_0} \right)^{\frac{1}{2}} \omega^2 (\epsilon^{\text{Hb}} - \epsilon_0)^2 R_{\text{D}}^{\text{Hb}} \quad (16)$$

and

$$\Delta\theta_{\text{W}} = \frac{1}{2} B \cdot S (\Delta\epsilon_{\text{r}})^{-\frac{1}{2}} \left(\frac{\mu_0}{\epsilon_0} \right)^{\frac{1}{2}} \omega^2 (\Delta\epsilon - \epsilon_0)^2 R_{\text{D}}^{\text{W}} . \quad (17)$$

At microwave frequencies, the permittivity of hemoglobin (ϵ^{Hb}) is about 2,^{2,8} which is the value characteristic of the (nonpolar) hydrocarbons.⁹ Where measurements exist for hemoglobin, the evidence suggests that there is no significant region of dispersion in dielectric constant between one Mc and optical frequencies, and presumably the value of the dielectric constant for the hemoglobin is only due to electronic polarization. Since the contribution of protein orientational polarization to the dielectric constant of hydrated hemoglobin is negligible at X-band frequencies, the mechanism responsible for the change in the dielectric constant of the hydrated hemoglobin is probably

due to dipole (orientational) polarization of water molecules on the hemoglobin.

To relate the measured $\Delta\theta_W$ with the free water dipole density, $\Delta\epsilon$ can be expressed in terms of the dipole density by using the Kirkwood theory for bound water in an equilibrium state.¹⁰ The Kirkwood theory deals with statistical mechanics, using electrostatic theory to account for long-range effects as well as short-range correlations. This theory has been successfully applied to calculate the dielectric constant of models of neighbor correlation by hydrogen bonds.¹¹ It is thus reasonable to apply this theory to the hydrated hemoglobin which has both intra- and inter- hydrogen bonds.

Since the dipoles of the adsorbed water molecules in the hemoglobin act as the principal polarization (only the dipoles of water are free to rotate at X-band frequencies), the dielectric contribution due to the presence of a small amount of water molecules can be derived from Kirkwood's equation:

$$\epsilon - \epsilon_{\infty} = g \frac{4\pi}{3kT} d^2 N_D, \quad (18)$$

where ϵ is the total dielectric constant of the medium, ϵ_{∞} is the ϵ at infinite frequency, d is the dipole moment of the water molecule, N_D is the dipole density, and g is Kirkwood's correlation factor for orientational effects between the central molecule and its neighbors.

The expression for $\Delta\epsilon$ can be obtained by introducing a model in which dipoles of water molecules are assumed to be embedded in a continuum of dielectric constant $\epsilon_\infty = \epsilon^{\text{Hb}}$. This assumption is reasonable because ϵ_∞ becomes ϵ^{Hb} in our model at X-band frequencies. Since $\epsilon = \epsilon^{\text{Hb}} + \Delta\epsilon - \epsilon_0$ in our model, the final expression becomes, from Eq. 18,

$$\Delta\epsilon - \epsilon_0 = g \frac{4\pi}{3kT} d^2 N_D . \quad (19)$$

We now express the Hall constant for the displacement current in water, R_D^W , in terms of free dipole concentration in analogy with the case of semiconductors.¹²

$$R_D^W = \frac{1}{N_D q_D} , \quad (20)$$

where N_D is the density of free dipoles in water, and q_D is the charge per pole of each electric dipole.⁶ We also define m as the correction factor which is a corresponding factor of $\frac{8}{3\pi}$ for silicon and germanium. ($\frac{8}{3\pi}$ is determined by the scattering effect from collisions of the carriers with lattice vibrations.) Then Eq. 20, with the correction factor, m , becomes

$$R_D^W = \frac{1}{m N_D q_D} , \quad (21)$$

where the correction factor, m , may depend on orientation of the bound water dipoles of different states.

It is difficult to derive the correction factor, m , exactly for the adsorbed water molecules of the hydrated hemoglobin because it is necessary to find the field existing within the bound water molecules in terms of the average field in the waveguide. Also, it is difficult to find the average field within randomly oriented dipoles of the unknown states in terms of the microscopic field that should be averaged over both the water molecules and the space between them. However, it is possible to obtain the value of m experimentally by measuring $\Delta\theta_W$, N_D , and $\Delta\epsilon$.

Although this approach involves simplified assumptions and a model, any exact or rigorous treatment of this problem presents formidable difficulties. The molecular structure of the hemoglobin is so complicated that one could hardly expect any exact treatment; rather, this crude approach is about all that is feasible to date.

Since we are only dealing with the case of small amounts of hydration,

$$g \cong 1,$$

and because $(\Delta\epsilon - \epsilon_0) < 1$,

$$\Delta\epsilon_r = \frac{\Delta\epsilon}{\epsilon_0} = 1 + \frac{(\Delta\epsilon - \epsilon_0)}{\epsilon_0} \cong 1.$$

Now substituting the expressions of Eqs. 20 and 19 into Eq. 17 and taking account that $g = 1$ and $\Delta\epsilon_r = 1$, we can express $\Delta\theta_W$ in terms of the dipole density N_D as:

$$\Delta\theta_W = \frac{B \cdot S \cdot \omega^2}{2m q_D} \left(\frac{\mu_0}{\epsilon_0} \right)^{\frac{1}{2}} \left(\frac{4\pi d^2}{3kT} \right)^2 N_D . \quad (22)$$

The relative change of the angle of rotation, $\Delta\theta_W$, is a function only of N_D at constant values of applied magnetic field B , temperature, and applied frequency. Measurements of $\Delta\theta_W$ in hemoglobin powder with a variable amount of water adsorbed can be applied to study the changes in bound water formation in hemoglobin molecules.

IV. EXPERIMENTAL

A. Sample Preparation

Twice-crystallized bovine hemoglobin (obtained from Calbiochem, Los Angeles, Calif.) was used as the protein sample, but it was not possible to estimate the absolute moisture content in the stock hemoglobin because the hemoglobin could not be completely dehydrated. Therefore, the relative water content was used. The weight fraction of the water content was expressed in terms of a difference between the weight of hydrated hemoglobin and that of the dry hemoglobin which was dried under vacuum for 20 hours over phosphorus pentoxide.

A saturated solution of lead nitrate, $\text{Pb}(\text{NO}_3)_2$ at 20°C , placed in a closed container produces 98% humidity. By placing known amounts of hemoglobin in such a container for various amounts of time, samples having different percentages of adsorbed water could be obtained. Since the hemoglobin was spread over a large surface area in the humid container, uniformity of hydration was assured.

The maximum hydration of the hemoglobin obtained by placing the sample in the 98% humid container was 26.5%. In order to obtain

samples within the range of the hydration values up to 35%, one more sample (higher than 26.5% hydration value) was prepared by circulating moist air into the sample. To prevent possible denaturization of the hemoglobin, fresh hemoglobin from stock (stored in a refrigerator) was used to prepare each sample. After weighing the sample to determine the percentage of water adsorbed, the grams of water per gram of hemoglobin were calculated by subtracting the amount of water. Necessary corrections were made to the measured hydration value of each sample to allow for atmospheric adsorption and evaporation of the sample.

The hemoglobin was placed in a lucite sample holder (cylindrical disks having a diameter of 2.2 cm and length of 1 cm). The average weight of hemoglobin in the sample holder was 400-450 mg. A lid was placed on the holder and an air-tight seal was made by ringing the lid with ethylene dichloride. Thus, samples could be placed in the waveguide and removed many times without altering the water content within the samples.

B. Angle Measurement

Techniques used to measure the angle of rotation have been described previously.¹³ Figure 1 is a block diagram of the detection system that uses a linear detection technique of the homodyne method.¹⁴

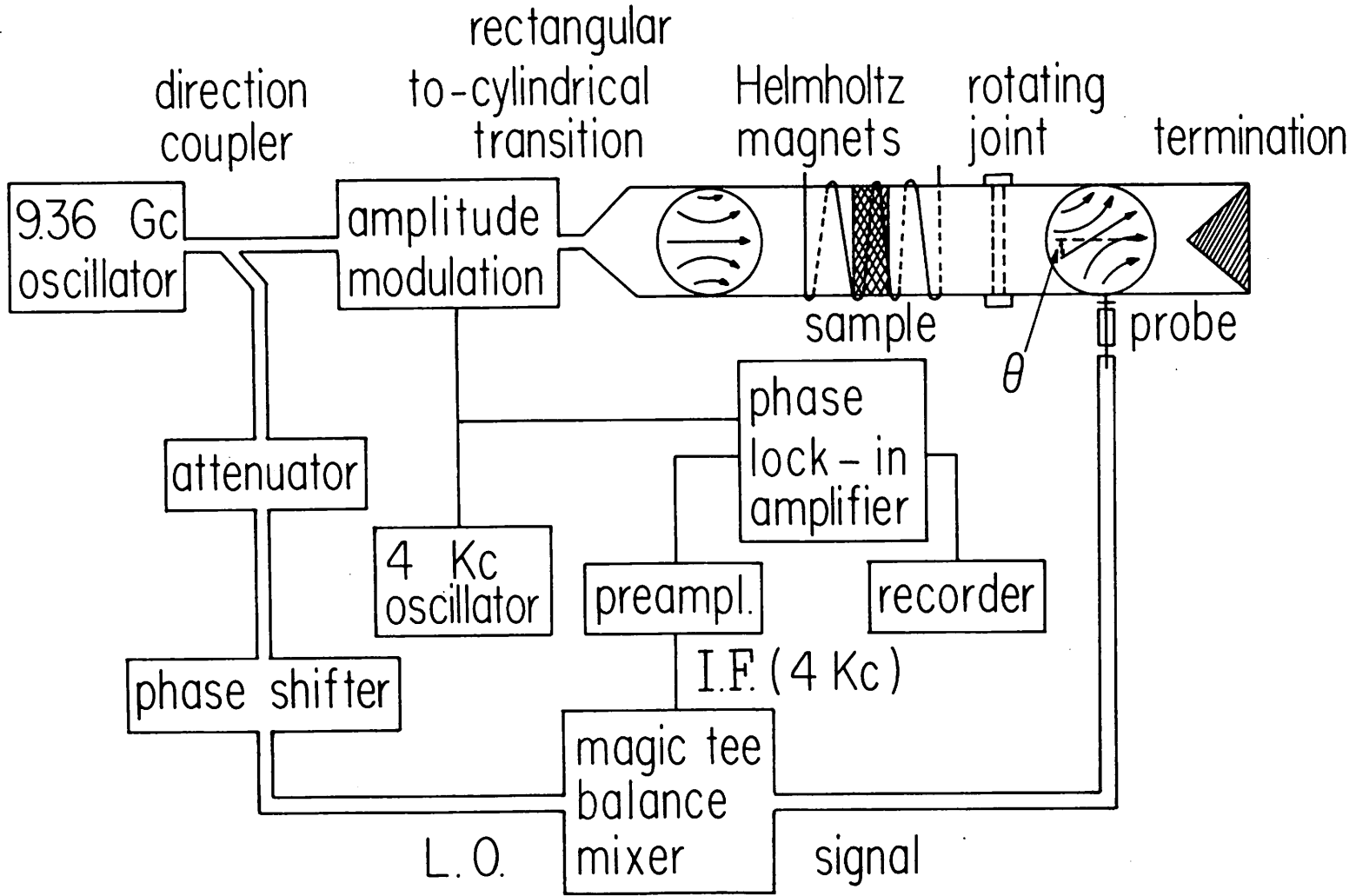


Fig. 1. Block diagram of detecting system.

The TE_{10} mode of 9.36 Gc signal (300 mW) is generated by an LFE oscillator model 814A and is converted to a TE_{11} circular mode. A rectangular X-band waveguide is used except for the section containing the sample and rotating joint. The sample is placed in a circular waveguide (2.3 cm i.d.) where alcohol of a constant temperature is circulating on the waveguide wall in order to maintain the sample at a constant temperature ($7^{\circ}C$). The magnet is Alpha Scientific Lab. Inc. model 352W Helmholtz coils, which provide a field strength up to 1500 G. Standing waves, which are likely to arise due to reflection at sample faces, can be reduced by using a slide-screw tuner. A piece of resistance card supported in polyfoam is placed within the circular waveguide between the sample and the rectangular-to-circular transition to maintain the well linearly polarized wave transmission. An amplitude modulated signal (from a gyrator), which contains information on Faraday rotation, is obtained by the microwave sampling probe located at $\theta = 90^{\circ}$. At this angle, the probe penetration disturbs the field least and the changes in the signal are most sensitive to the rotation. Since the probe is in a practically linear portion of the $\cos \theta$ distribution of the electric field, the probe voltage is calibrated with the rotation of the angle by reflecting light from an attached mirror to the rotating section of the circular waveguide.

The output signal (4 Kc) of the mixer is connected to a low-noise preamplifier (Princeton Applied Research Corp., model CR-4A)

through a matching transformer (PAR model AM-1). The preamplifier output is then fed to the PAR phase lock-in amplifier model JB-4. With this system, an angle of approximately 10^{-4} degree rotation can be detected and the sign of the output signal from the amplifier (JB-4) depends on the direction of the rotation.

C. Results

The detection system was first calibrated by placing the probe initially at $\theta = 90^\circ$ such that the output from the probe is zero. Then the probe was rotated to a known angle and the corresponding probe output voltage was recorded. The linear relationship between the output voltage from the probe and the angular position of the probe was found to be within the angle of about a half degree each side of $\theta = 90^\circ$. The reliability of the calibration method was checked by using the n-type silicon wafer of 15 ohm-cm resistivity (0.5 mm thick), which in a magnetic field of 1000 G, exhibited 0.22 degree rotation. This angle is in excellent agreement with that obtained by theoretical calculation.⁵

X-band Faraday rotation experiments were performed in a dc magnetic field of 1500 G, at a sample temperature of 7°C and a frequency of 9.36 Gc. Nine samples, each 1 cm thick and containing a different percentage of adsorbed water, were investigated. The experimental results were plotted in Fig. 2 as rotation in degrees

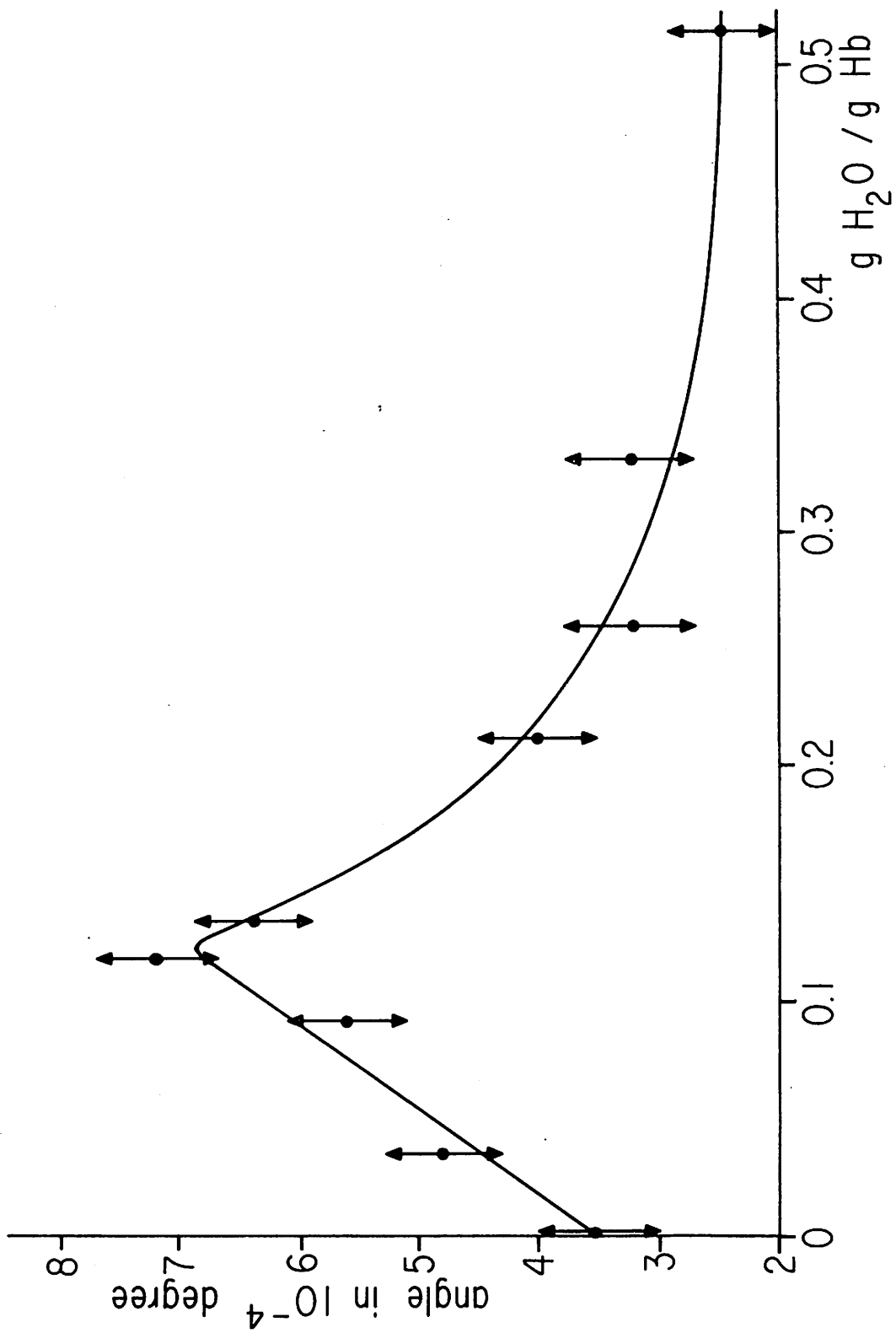


Fig. 2. Angle of rotation versus hydration in grams of water per gram hemoglobin.

versus amounts of the adsorbed water in grams of water per gram of hemoglobin. The vertical bar through each data point represents the uncertainty. The uncertainty involves angle measurement errors, 5 to 10 percent errors in interpretation of the recorder charts (angle resolution of about 10^{-4} degrees), and an error in the measurement of the water content in the sample. Since this experiment extended over a short period of time, changes in sample temperature were not observed.

V. DISCUSSION AND CONCLUSION

The result (Fig. 2) shows that the angle of rotation increases linearly with the amount of adsorbed water until the hydration level reaches about 0.13 grams of water per gram of dry hemoglobin. The angle of rotation decreases as the water content increases above this water concentration. This indicates that formation of the hydration layer begins at this concentration by forming hydrogen-bonded clusters of water molecules on the surface of hemoglobin because the tightly bound water molecules are no longer free to rotate at microwave frequencies.

We define the hydration level of 0.13 grams of water per gram of dry hemoglobin as a critical hydration which indicates the changes in structures of bound water. This value of hydration (0.13) is lower than the value (0.24) obtained from the study of dielectric properties by Rosen.² The difference between these two values is believed to be due to the different reference of the dry sample because the hemoglobin sample can not be dried completely without denaturing it. From Fig. 2, we can obtain about 0.05 grams of water per gram of hemoglobin in the dry sample by extrapolating the linear portion of the curve. The hydration value of 0.18 grams of water per gram of hemoglobin,

obtained by adding 0.05 to our value, is somewhat closer to Rosen's value. It is, however, not necessary to have the same value because Rosen used protein samples other than hemoglobin.

From the studies of dielectric dispersion,^{2,8,15} the hemoglobin shows no significant region of dielectric dispersion above the frequencies higher than 1 Mc. Therefore, it can be safely assumed that contributions to the dielectric constant from hemoglobin orientational polarization are negligible at microwave frequencies. Since the dielectric constant of hemoglobin itself does not change at microwave frequencies, the frequency dependence of the dielectric constant of the hydrated hemoglobin is strongly dependent on the amount of adsorbed water. The dipolar relaxation of free water molecules shows a Debye type relaxation in the range 10^9 to 10^{11} cycles per second,¹⁶ so that its relaxation plays a prominent part in the dielectric constant increase of the hydrated hemoglobin at the microwave region.

Relating the dielectric constant with free dipole density, we are able to express the angle of Faraday rotation as a function of free water molecules present in the hydrated hemoglobin (Eq. 22). The experimental data are indeed found to be linearly proportional to the density of free water dipoles within the region below the critical hydration, as predicted by Eq. 22, which is applicable only when the water molecules are free to rotate at X-band frequencies (up to the critical hydration). It is also of interest to note that the measurement

of the Faraday rotation versus the magnetic field shows linear dependence. This result is further proof of the validity of Eq. 22.

As the amount of adsorbed water increases beyond the critical hydration, the free water molecules (monomers which are bound by hydrogen bonds to the polar groups of hemoglobin, such as $-NH_2$ and $-COOH$) start to form hydrogen-bonded clusters (dimers, trimers, etc.) and result in an ice-like structure on the surface of the hemoglobin. Since the frequency dependence of the dielectric constant of ice shows that water molecule dipoles in ice structure undergo relaxation at frequencies somewhere near $10 Kc$,⁴ the water molecules bound into an ice-like structure on the hemoglobin are unable to orient as the free water molecule at microwave frequencies. In this case, bound water appears "irrotational" at high microwave frequencies.

A qualitative theory about the effect of water on a hemoglobin surface is still lacking, but it is believed that this experiment confirms the theory which is based on the dipole relaxation of the bound water.

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REFERENCES

1. B. Rosenberg, J. Chem. Phys. 36, 816 (1962).
2. D. Rosen, Trans. Faraday Soc. 59, 2178 (1963).
3. S. Takashima, J. Polymer Sci. 62, 233 (1962).
4. H. Granicher, C. Jaccard, P. Scherrer, and A. Steinemann, Discussions Faraday Soc. 23, 50 (1957).
5. R. Rau and M. Caspari, Phys. Rev. 100, 632 (1955).
6. H. Barlow, IEE Monograph 431E, 349 (1961).
7. M. Engineer and B. Nag, J. Appl. Phys. 36, 3387 (1965).
8. H. Schwan, Advances in Biological and Medical Physics (Academic Press, New York, N. Y., 1957), Vol. V, p. 147.
9. C. Böttcher, Theory of Electric Polarisation (Elsevier Publishing Co., New York, N. Y., 1952), p. 329.
10. J. Birks and J. Hart, Progress in Dielectrics (Heywood & Co. Ltd., London, England, 1961), Vol. III, p. 68.

11. J. Kirkwood and J. Shumaker, Proc. Natl. Acad. Sci. U. S. 38, 855 (1952).
12. W. Shockley, Electrons and Holes in Semiconductors (Van Nostrand Co., Inc., New York, N. Y., 1950), p. 250.
13. S. Y. Chai and P. O. Vogelhut, accepted by Rev. Sci. Instr. for publication.
14. D. Ring, Bell Lab. Record 34, 465 (1956).
15. J. Roberts and H. Cook, Brit. J. Appl. Phys. 3, 33 (1952).
16. F. Buckley and A. Moryott, U. S. Natl. Bur. Std. Circular 589, 1 (1958).