An Investigation by Dielectric Methods of Hydration in Myoglobin Solutions

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The permittivities of three solutions of sperm-whale myoglobin of different concentrations were measured in the frequency range 300–1300 MHz at 20°C by using a coaxial-line technique. These results were combined with those measured previously at frequencies below 10 MHz. Two methods are described for calculating the extent of macromolecular hydration from the data. The more reliable method yields results of approx. 0.25 g of H₂O/g of protein, which is in satisfactory agreement with the theoretically calculated value. Agreement with the value found from the rotational motion of the molecule is not so close, which is probably caused by the different meanings that may be ascribed to the term hydration.

Aqueous solutions of proteins have been subjected to dielectric analysis for many years; most of this work has been in the radio-frequency region up to about 10 MHz, since it is here that the β dispersion of the system occurs and may be readily measured by using alternating-current bridge techniques. For the smaller proteins the original contention of Oncley (1943) that the β dispersion arises from the Brownian rotation of the molecular dipole moment has been verified (South & Grant, 1972; Hendrickx *et al.*, 1968).

Some measurements have been also made at very much higher frequencies (around 1 GHz) and it is these that are particularly valuable in the investigation of macromolecular hydration. There are two ways in which the presence of such water of hydration may be reflected in the high-frequency permittivity of the solution. Both use the fact that dielectric measurements are able to distinguish between processes having different fluctuation times, since the faster processes show dispersion at higher frequencies; at frequencies around 1 GHz pure water is readily able to follow the oscillating field but rotation of the macromolecule or of its hydration is not possible. In the first method the permittivity of the solution is found to be less than that calculated by assuming that the volume occupied by the protein behaves as a vacuum, i.e. has the minimum possible permittivity. This indicates that there is less free water in the solution than the total quantity of water known to be present; the difference is attributed to water of hydration which, at this frequency, exhibits a low relative permittivity (possibly approx. 5) instead of the value appropriate to free water (approx. 80 at 20°C).

A second manifestation of hydration is found when these high-frequency results are compared with the radio-frequency measurements mentioned above.

A small dispersion is found which extends from about 10 MHz to 1 GHz and which may be attributed to the bound water. The situation is illustrated in Fig. 1 where the protein, free-water and bound-water dispersion are designated β , γ and δ respectively.

Studies such as these have been carried out on a variety of proteins by Buchanan *et al.* (1952), on human serum albumin by Grant (1957), on haemoglobin by Schwan (1957, 1965) and by Pennock & Schwan (1969), on egg albumin by Grant (1962, 1965) and on bovine serum albumin by Grant *et al.* (1968).

One obvious exception from this list of studies is that of myoglobin, which is a simple protein of molecular weight 17000 whose structure has been thoroughly investigated by X-ray crystallography (Kendrew *et al.*, 1961) and whose β dispersion is

Fig. 1. Complete dispersion curve for a typical protein solution

β , γ and δ are dispersion curves for protein, free water and bound water respectively at 20°C. See the text for further details.

also well documented and understood (South & Grant, 1972). It is the purpose of the present paper to investigate whale myoglobin solutions at frequencies higher than those pertaining to the β dispersion to obtain estimates of the extent of hydration.

Measurements and Analysis of Results

Three solutions of sperm-whale myoglobin (produced by Seravac Ltd., Holyport, Maidenhead, Berks., U.K.) were measured at frequencies between 300 and 1300 MHz. These samples were prepared and electrolysed in the same way as previously described (South & Grant, 1972). The concentrations were measured by a spectrophotometer, and found to be 77, 99 and 161 mg/ml. The value of the extinction coefficient ($E_{280} = 18.7$) was provided by the supplier of the myoglobin.

All dielectric measurements were made at 20°C in

a coaxial-line cell (Grant & Keefe, 1968) by using a standing-wave method which has been described previously (Sheppard, 1972; Sheppard & Grant, 1972). The results are shown in Table 1 and in Fig. 2.

Table 1. *Relative permittivity (ϵ') at 20°C of myoglobin solutions of concentration 77, 99 and 161 mg/ml (a, b and c respectively)*

The errors are the 95% confidence intervals.

Frequency (MHz)	ϵ'		
	a	b	c
320	74.5 $\pm$ 0.4	72.5 $\pm$ 0.1	68.9 $\pm$ 0.2
470	73.9 $\pm$ 0.4	72.3 $\pm$ 0.1	68.4 $\pm$ 0.3
600	73.6 $\pm$ 0.3	72.2 $\pm$ 0.2	68.2 $\pm$ 0.3
800	73.8 $\pm$ 0.4	71.9 $\pm$ 0.2	67.7 $\pm$ 0.1
1050	72.8 $\pm$ 0.4	70.7 $\pm$ 0.1	67.4 $\pm$ 0.1
1260	72.0 $\pm$ 0.6	70.4 $\pm$ 0.3	—

Fig. 2. *Experimental dispersion curve for myoglobin solutions of concentrations 7.7% (▲), 9.9% (■) and 16.1% (●)*
The points below 10 MHz were obtained by interpolation of previously published data (South, 1970).

Table 2. δ dispersion parameters for myoglobin

$\epsilon_{s\delta}$ was obtained from eqn. (1) ($\epsilon_{s\delta} = \epsilon_{\infty\delta}$). $\epsilon_{\infty\delta}$ was obtained by extrapolating the free-water (γ) dispersion to low frequencies ($\epsilon_{\infty\delta} = \epsilon_{s\gamma}$).

Concentration	$\epsilon_{s\delta}$	$\epsilon_{\infty\delta}$
77	77.0	73.9 ± 0.6
99	76.1	72.4 ± 0.5
161	73.6	68.5 ± 0.5

As pointed out above, it is useful to compare the high-frequency results with those measured at radio frequencies. To achieve this the data of South (1970) have been used. Unfortunately the temperatures and concentrations chosen by South (1970) do not coincide with those of the present study; however, the radio-frequency data had been measured over a wide range of temperature and concentration, and by constructing graphs of permittivity against these variables at each frequency we have interpolated the values appropriate to 20°C and to the three concentrations 77, 99 and 161 mg/ml. These complete sets of data are shown in Fig. 2.

The parameters required to calculate the degree of hydration by the two methods described above are the permittivities of the high- and low-frequency limits of the δ dispersion as shown in Fig. 1. It is evident from Fig. 2 that the high-frequency limit $\epsilon_{\infty\delta}$ is not exactly defined by the measurements, since the δ dispersion merges slightly with the low-frequency end of the dispersion caused by the free water; this is particularly so for the highest concentration measured. In establishing the value of $\epsilon_{\infty\delta}$ it has therefore been necessary to estimate the contribution of pure water; this has been done by using the well-known fact that the low-frequency part of the water dispersion may be represented to a very good approximation by a Debye dispersion curve. The general form of such a curve is

$$\epsilon - \epsilon_{\infty} = \frac{\epsilon_s - \epsilon_{\infty}}{1 + x^2} \quad (1)$$

where ϵ_s and ϵ_{∞} are the respective permittivities at the low- and high-frequency ends of the dispersion region. The parameter x may be written $x = \omega\tau = f/f_R$, where τ and f_R are the relaxation time and relaxation frequency of water respectively; f is the frequency and $\omega = 2\pi f$. The highest frequency used in the present work was 1.26 GHz and the value of f_R is around 17 GHz, so at our three highest frequencies f^2 is much greater than f_R^2 and eqn. (1) comes down to a straight-line relationship between ϵ and f^2 with an intercept of ϵ_s on the ϵ axis. Hence to obtain values for $\epsilon_{s\gamma} = \epsilon_{\infty\delta}$ graphs of permittivity were drawn against f^2 for each concentration at the three highest frequency

points and were then extrapolated to give the intercept. At the lowest concentration the measurement of ϵ was repeated at the highest frequency and the value of ϵ used for the extrapolation was that quoted in Table 1, which is the mean of the values shown in Fig. 2. This procedure gave the values shown by the lower set of dashed lines in Fig. 2 and in the final column of Table 2.

The low-frequency limit of the δ dispersion, $\epsilon_{s\delta}$, is found by extrapolating the low-frequency dispersion data to its high-frequency limit, $\epsilon_{\infty\delta}$, and this is equated to $\epsilon_{s\delta}$. The β dispersion is known to be well represented as a single Debye process and this extrapolation can therefore be made by using a procedure that fits the data to the Debye expression referred to by eqn. (1). In this case it is more convenient to write $\epsilon_s - \epsilon_{\infty} = \Delta_\beta$ and $x = \omega\tau_\beta$, where ω is the angular frequency of the applied field, Δ_β is the magnitude of the β dispersion and τ_β is its relaxation time. This analysis leads to the results shown in Table 2.

Interpretation

To interpret the data summarized in Table 2 it is necessary to assume a model which is, at the same time, both reasonably realistic and tractable. The model for haemoglobin used by Pennock & Schwan (1969) will also be used here. It consists of a sphere of protein of permittivity ϵ_p , surrounded by a spherical shell of water of hydration with permittivity ϵ_h . This composite particle is suspended in solvent water having a permittivity ϵ_w . Some justification of this model is necessary, since it is clearly a simplification of the real system. The model assumes a spherical shape for the protein; this is not too far from reality, since the X-ray-crystallography analysis indicates molecular diameters of $2.3 \text{ nm} \times 3.5 \text{ nm} \times 4 \text{ nm}$ (Kendrew *et al.*, 1958) and the β dispersion is very close to a single relaxation time. The hydration shell is considered to be uniform, whereas in reality both the quality and quantity of hydration depend on the nature of the neighbouring macromolecular surface: the myoglobin surface is, however, reasonably uniform; it contains about 50 charged groups and these are distributed in a regular way; there are no areas containing a predominantly large or small number of such groups. The water of hydration is represented by a bulk permittivity ϵ_h , which it will be assumed varies from 100 at the lower-frequency end of its dispersion (South & Grant, 1972; Grant *et al.*, 1971; Schwan, 1965) to 5 at the high-frequency end, the frequency range of this dispersion being between 100 and 500 MHz; the hydration shell is not thick enough for these values to be considered as 'the permittivity of water of hydration' but are to be thought of merely as effective parameters. The model assumes that the permittivity of the protein

itself is independent of frequency up to at least 1 GHz; this is justified since this permittivity, whose value will be taken as 5, arises from perturbations in electronic and nuclear wave functions and which show resonance behaviour at very much higher frequencies than 1 GHz (the highest frequency encountered in the present work). The permanent dipole moment of the protein is not included because the rotational correlation time of the protein is very much longer than the reciprocal of the frequencies at which ϵ_{δ} and $\epsilon_{\infty\delta}$ are measured. This dipole moment therefore plays no part in the electrostatic interactions at these frequencies apart from inducing further dipoles within the protein and hydration shell which also rotate with the long correlation time and are therefore not discernible above 10 MHz.

The effective permittivity of the protein plus water of hydration, ϵ_p , may be calculated from standard electrostatic theory, i.e. by solving Laplace's equation subject to the relevant boundary conditions, which yields the result:

$$\frac{\epsilon_p - \epsilon_h}{\epsilon_p + 2\epsilon_h} = \frac{1}{1 + w'} \frac{\epsilon_p - \epsilon_h}{\epsilon_p + 2\epsilon_h} \quad (2)$$

where w' is the ratio of the volume of water of hydration to the volume of the protein; it is therefore related to the more usual weight-fraction measure of the water of hydration, w , by

$$w' = \frac{\rho_p}{\rho_h} w \quad (3)$$

where ρ_p and ρ_h are the densities of protein and hydration shell respectively; ρ_h is assumed to be 1 g/cm³ and ρ_p may be taken as the reciprocal of the partial specific volume of the protein, which for myoglobin is 1.35 g/cm³. An alternative method of evaluating ρ_p might be to divide the molecular mass by the volume deduced from the molecular dimensions stated above, assuming an ellipsoidal shape for the molecule. Although this simplifying assumption is clearly open to criticism it is interesting to see the effect of ρ_p on the calculated value of the degree of hydration w . With this method $\rho_p = 1.52$ g/cm³ which, although being 12% different from the value of ρ_p obtained from the partial specific volume, changes the value of w in the third decimal place only if w is calculated from eqns. (7)–(10). If, however, w is calculated from the value of $\epsilon_{\infty\delta}$ (eqn. 6) rather than from the magnitude of the δ dispersion the effect of the choice of the value of ρ_p is considerable. This is made clear in Table 3 where it is seen that a negative value for the degree of hydration can be obtained which is physically inadmissible.

The value of the permittivity of the solution, ϵ , must be expressed in terms of ϵ_p , ϵ_w and the number of hydrated solute particles per unit volume of the solution. In general this poses a very difficult problem

in view of the interactions between neighbouring protein molecules. Fortunately the solutions measured are sufficiently dilute that the effect of these interactions is very small. If they were ignored completely the mixture formula of Sillars (1937) would be obtained. However, the solutions are sufficiently concentrated for subsidiary calculations to show that this effect should be taken into account to a first approximation. To do this the solute particle is assumed to be surrounded by a shell of solvent with a volume equal to the volume of solvent per solute particle in the whole solution; this shell of solvent is then surrounded by a medium whose permittivity is equal to that of the solution. By standard electrostatics again this results in

$$\frac{\epsilon - \epsilon_w}{\epsilon + 2\epsilon_w} = p \frac{\epsilon_p - \epsilon_w}{\epsilon_p + 2\epsilon_w} \quad (4)$$

where p is the volume fraction of solute in the system. This is the formula first derived by Maxwell (1892). Thus if C is the concentration in g·cm⁻³, the volume fraction of the protein is C/ρ_p and thus

$$p = \frac{(1 + w') C}{\rho_p} \quad (5)$$

The evaluation of w' (or w) therefore comes down to the solution of eqns. (2) and (4). There are two independent ways of doing this; the first uses $\epsilon_{\infty\delta}$ only and the other uses $(\epsilon_{\delta} - \epsilon_{\infty\delta})$, i.e. the magnitude of the δ dispersion.

(a) Calculation of w from $\epsilon_{\infty\delta}$

Since at the high-frequency end of the δ dispersion ϵ_p and ϵ_h are small compared with ϵ_w and $\epsilon_{\infty\delta}$ it follows that $\epsilon_p \ll \epsilon_w$ and hence eqn. (4) may be simplified to

$$\frac{\epsilon_{\infty\delta} - \epsilon_w}{\epsilon_{\infty\delta} + 2\epsilon_w} = -\frac{1}{2}p \quad (6)$$

Table 3. Hydration values for myoglobin

Concentration (mg/ml)	w (g of H ₂ O/g of protein)		
	Calculated from $\epsilon_{\infty\delta}$		Calculated from Δ_δ
	*	†	‡
77	-0.01 ± 0.07	0.05 ± 0.07	0.27 ± 0.06
99	-0.02 ± 0.03	0.03 ± 0.05	0.27 ± 0.04
161	-0.06 ± 0.03	0.00 ± 0.03	0.23 ± 0.03

* Values calculated by using eqn. (6) with $\rho_p = 1.35$.

† Values calculated by using eqn. (6) with $\rho_p = 1.52$.

‡ Values calculated by using eqns. (7)–(10).

from which p is readily found. ρ_p is found from the molecular weight and the volume of the molecule established from X-ray crystallography and hence w' (and w) may be found for each concentration from eqn. (5). The results are shown in Table 3.

(b) *Calculation of w from the magnitude of the δ dispersion*

Eqns. (2) and (4) each give rise to two equations:

$$\frac{\epsilon_{sp} - \epsilon_{sh}}{\epsilon_{sp} + 2\epsilon_{sh}} = \frac{1}{1 + w'} \frac{\epsilon_p - \epsilon_{sh}}{\epsilon_p + 2\epsilon_{sh}} \quad (7)$$

$$\frac{\epsilon_{\infty p} - \epsilon_{\infty h}}{\epsilon_{\infty p} + 2\epsilon_{\infty h}} = \frac{1}{1 + w'} \frac{\epsilon_p - \epsilon_{\infty h}}{\epsilon_p + 2\epsilon_{\infty h}} \quad (8)$$

$$\frac{\epsilon_{sd} - \epsilon_w}{\epsilon_s + 2\epsilon_w} = \frac{c(1 + w')}{\rho_p} \frac{\epsilon_{sp} - \epsilon_w}{\epsilon_{sd} + 2\epsilon_w} \quad (9)$$

$$\frac{\epsilon_{\infty d} - \epsilon_w}{\epsilon_{sd} + 2\epsilon_w} = \frac{c(1 + w')}{\rho_p} \frac{\epsilon_{\infty p} - \epsilon_w}{\epsilon_{\infty d} + 2\epsilon_w} \quad (10)$$

The subscripts s and ∞ refer respectively to low- and high-frequency limiting values.

An expression for $\Delta_\delta (= \epsilon_{sd} - \epsilon_{\infty d})$ may be found from eqns. (9) and (10) and then eqns. (7) and (8) used to eliminate ϵ_{sp} and $\epsilon_{\infty p}$. In general this leads to some cumbersome algebra and the solution of these simultaneous equations has therefore been done by computer. The resulting values of w are shown in Table 3.

Discussion

It may be seen in Table 3 that there is some discrepancy between the degrees of hydration calculated by the two methods. The values found from Δ_δ are in good agreement with the theoretical result of 0.29 g of H_2O/g of protein calculated by Fisher (1965) on the basis that each surface polar group is surrounded by a monomolecular layer of hydration and that each such group is 0.4 nm thick. They are also consistent with the value of 0.22 g of H_2O/g of protein found by Rosen (1963) by dielectric measurements of dampened myoglobin powders. On the other hand the very low values of w found from $\epsilon_{\infty d}$ seem to be unrealistic. This is not entirely surprising since this method of calculation suffers from two weaknesses. First, it is extremely sensitive to the value assumed for the protein density; for instance changing ρ_p by 10% can result in a change in w of a factor of 2 or 3. Secondly, the water of hydration is responsible for only a very small part of the quantity $(\epsilon_w - \epsilon_{\infty d})$ which is used in the calculation; any small inadequacy of the mixture formula therefore results in large errors in w . We conclude that this method of assessing w is considerably less reliable than the former one, which has the advantages of being very insensitive to ρ_p and of making use of a quantity (Δ_δ) that is almost directly proportional to w .

The degrees of hydration obtained here from Δ_δ are lower, by a factor of about one-half, than the estimates found from analysis of the β dispersion (South & Grant, 1972) even allowing for the most extreme shape that seems possible for the myoglobin molecule. This emphasizes the simple nature of the model and the disagreement could be caused by the methods measuring different types of hydration. The β dispersion assesses the water that rotates with the molecule (i.e. mechanically different from free water), whereas the two methods presented here measure water that has a different electrical behaviour from free water; these two quantities of water could not, in general, be expected to be equal. Another possible reason for this difference is that the calculation of w from the relaxation time of the β dispersion, τ_β , requires the use of the formula

$$\tau_\beta = \frac{4\pi a^3 \eta}{kT} \quad (11)$$

where η is the viscosity of the medium in which the macromolecule rotates, kT is the thermal energy and a is the radius of the hydrated molecule. In the calculation η is assumed to be equal to the bulk viscosity of the solvent water (τ_β is extrapolated to zero concentrations); however, the form of the boundary between the water and the macromolecule may mean that the appropriate viscosity is different from the bulk value. The irregular nature of the protein surface may, for instance, introduce greater friction than appropriate for the 'ball-bearing' model on which eqn. (11) is based; this would create a larger relaxation time than expected and would therefore decrease the calculated amount of bound water.

Both methods of calculating w presented here seem to indicate a slightly lower degree of hydration at high concentration. This could be caused by the mixture formula taking insufficient account of protein-protein interactions, but it is also possible that the proximity of protein molecules does affect their interaction with the solvent, and clearly, since there is only a finite amount of water in the system, the degree of hydration of each protein molecule must surely fall at some stage as the concentration is increased. This question must await further investigation.

It has been assumed throughout that the δ dispersion is caused by the relaxation of a hydration shell. There are, in principle, other possible causes of this dispersion, notably proton fluctuation and the rotation of surface polar groups (Pennock & Schwan, 1969; South & Grant, 1972). The evidence presented here is not intended as proof that the hydration mechanism is the correct one but merely as a further indication that this assumption does produce a consistent interpretation of the results.

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References

- Buchanan, T. J., Haggis, H. G., Hasted, J. B. & Robinson, B. G. (1952) *Proc. Roy. Soc. Ser. A* **213**, 379–391
- Fisher, H. F. (1965) *Biochim. Biophys. Acta* **109**, 544–550
- Grant, E. H. (1957) *Phys. Med. Biol.* **2**, 17–28
- Grant, E. H. (1962) *Nature (London)* **196**, 1194–1195
- Grant, E. H. (1965) *Ann. N. Y. Acad. Sci.* **125**, 418–427
- Grant, E. H. & Keefe, S. E. (1968) *Rev. Sci. Instrum.* **39**, 1800–1803
- Grant, E. H., Keefe, S. E. & Takashima, S. (1968) *J. Phys. Chem.* **73**, 4373–4380
- Grant, E. H., South, G. P., Takashima, S. & Ichimura, H. (1971) *Biochem. J.* **122**, 691–699
- Hendrickx, H., Verbruggen, R., Rosseneu-Mottreff, M. Y., Blaton, V. & Peeters, H. (1968) *Biochem. J.* **110**, 419–424
- Kendrew, J. C., Bodo, G., Dintzis, H. M., Parrish, R. G., Wyckoff, H. & Phillips, D. C. (1958) *Nature (London)* **181**, 662–666
- Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C. & Shore, V. C. (1961) *Nature (London)* **190**, 666–672
- Maxwell, J. C. (1892) *A Treatise on Electricity and Magnetism*, Oxford University Press, Oxford
- Oncley, J. L. (1943) in *Proteins, Amino-Acids and Peptides* (Cohn, E. J. & Edsall, J. T., eds.), chapter 22, Reinhold Publishing Corporation, New York
- Pennock, B. E. & Schwan, H. P. (1969) *J. Phys. Chem.* **73**, 2600–2610
- Rosen, D. (1963) *Trans. Faraday Soc.* **59**, 2178–2191
- Schwan, H. P. (1957) *Advan. Biol. Med. Phys.* **5**, 147–209
- Schwan, H. P. (1965) *Ann. N. Y. Acad. Sci.* **125**, 344–354
- Sheppard, R. J. (1972) *J. Phys. D* **5**, 1576–1587
- Sheppard, R. J. & Grant, E. H. (1972) *J. Phys. E* **5**, 1208–1212
- Sillars, R. W. (1937) *J. Inst. Elec. Eng.* **80**, 378–394
- South, G. P. (1970) Ph.D. Thesis, University of London
- South, G. P. & Grant, E. H. (1972) *Proc. Roy. Soc. Ser. A* **328**, 371–387