A STREAMING DIELECTRIC INVESTIGATION OF HELIX POMATIO HEMOCYANIN

by

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Previously a streaming dielectric study has been made of sodium thymonucleate[1] and sodium hyaluronate[2]. It was shown that the dielectric increment observed in aqueous solutions of these substances cannot possibly be due to a rotation of the whole macromolecules in the electric field. These results cast some doubt on the generally made assumption that the dielectric properties of high molecular weight compounds arise from rotations of the macromolecules.

In order to investigate this problem further a streaming dielectric investigation has been made of Helix Pomatia hemocyanin. This protein is particularly suitable for testing dielectric theories since it can easily be obtained in pure form, and also, since it has been carefully studied by other methods, its molecular dimensions are therefore better known than for most other proteins.

EXPERIMENTAL

Hemocyanin was prepared according to the method of BROHULT[3] from hibernating Helix Pomatia snails. The hemocyanin solutions obtained were dialyzed three times at 4° C with distilled water for about four hours each time. The electrolyte-free solutions thus obtained were treated in three different ways. In most cases the solutions were immediately used for measurements in the streaming dielectric apparatus (prep. A). In some cases (prep. B) the solutions were stored for four days at 4° C and the precipitate formed (*cf.* BROHULT[3] p. 15) was removed by centrifugation before measurements. A few experiments were also made on a freeze-dried preparation (prep. C) made from the dialyzed solutions. When the freeze-dried hemocyanin was redissolved a small insoluble portion had to be removed by centrifugation.

The concentrations of the solutions were determined by nitrogen analysis according to Kjeldahl. The nitrogen content of hemocyanin was taken as 15.15 %[3].

Hemocyanin solutions of various concentrations were studied in the streaming dielectric apparatus previously described[1]. In this apparatus the solutions to be investigated are placed in the annular space between two concentric cylinders forming the measuring cell. The inner cylinder is rotated with various speeds to give a velocity gradient between 0 and 10,000 $\sec^{-1}$ in the cell where the electric field is perpendicular to the stream lines, and between 0 and 3,600 $\sec^{-1}$ in the cell where the electric field is parallel with the stream lines. The dielectric constant and the conductance of the solutions subjected to these velocity gradients were determined between 0.1 and 6 Mc. at 20° C.

To enable calculation of critical velocity gradients[1] (G_c) the viscosity of the solutions were measured with an Ostwald viscosimeter. The reaction of each solution was determined with a glass electrode pH-meter before each streaming dielectric experiment. The meter was calibrated before each measurement.

RESULTS

Two types of measurements have been made: one where the electric field is perpendicular to the stream lines and the other where the two fields are mainly parallel. The former type of measurement is the one of main interest. With the parallel field cell the exact electric field conditions are unknown and therefore no quantitative data can be obtained[1].

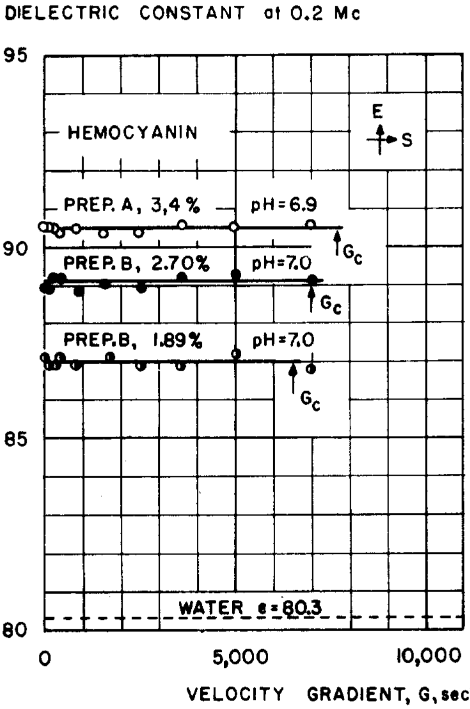

Fig. 1. Streaming dielectric results for hemocyanin at 20° C. Electric field perpendicular to stream lines. Concentration in gram per 100 ml solution. G_c is the critical velocity gradient for laminar flow.

In the adjoining figures the results are shown for the measurements where the electric field was perpendicular to the stream lines. In Fig. 1 some typical data are given for the dielectric constant at 0.2 Mc as a function of the velocity gradient. In no case was a streaming dielectric effect observed, that is there was no change of dielectric constant or conductance with the velocity gradient. All three preparations of hemocyanin were studied at concentrations between 0.7 and 2.7 g hemocyanin per 100 ml of solution. The dielectic increment per gram of hemocyanin decreased slightly with increasing concentration. From low concentration data a value of 3.7 dielectric units per g of hemocyanin per 100 ml of solution was calculated.

Whereas the absolute accuracy of the measured dielectric constant was ± 0.3 dielectric units, the accuracy with which a streaming dielectric effect could be observed was better than 0.1 dielectric units. This is because values for the impedance of the measuring cell always were determined at zero velocity gradient immediately before and after measurements at a given velocity gradient. By this procedure errors such as those due to temperature changes in the measuring cell are eliminated.

In Fig. 2 some dispersion curves are shown for various concentrations of hemocyanin. Since the dielectric increment was independent of the velocity gradient in the solutions, the dispersion frequencies were also. The critical frequency varied from 0.69 Mc for dilute solutions (0.78%) to 1.1 Mc for concentrated solutions (3.1%). At high frequencies a dielectric decrement was observed amounting to approximately half a dielectric unit per g hemocyanin per 100 ml of solution. An exact determination of the high frequency decrement was, however, not possible since the accuracy of the bridge method used is highly reduced above 7 Mc.

In the experiments where the electric field was parallel to the stream lines a small streaming dielectric effect was observed at frequencies below 1 Mc. The dielectric increment increased slightly with increasing velocity gradient. The frequency of the dispersion was, however, independent of the velocity gradient. The accuracy of these measurements is low and the electric field conditions are non-ideal; therefore no large emphasis should be placed on the small increases observed. It is of interest to note, however, that no decrease in dielectric constant with increasing velocity gradient was ever observed.

No difference in dielectric properties was observed for the hemocyanin preparations A and B. However, preparation C, which gave highly viscous solutions and showed other signs of denaturation, had much higher (2 to 5 times) increments per g substance than the preparations A and B.

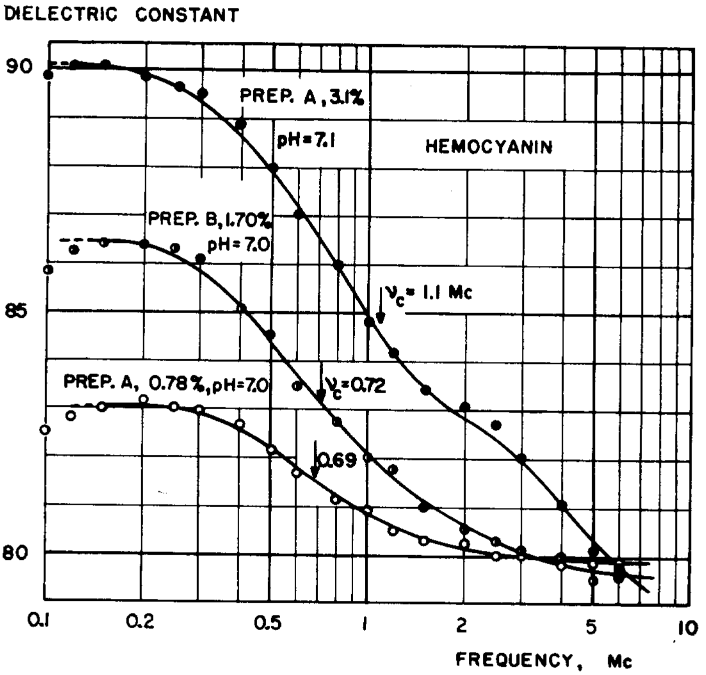

Fig. 2. Dispersion curves of hemocyanin at 20° C. Critical frequencies (v_c) indicated by arrows.

DISCUSSION

The properties of the Helix Pomatia hemocyanin molecule have been carefully studied by many different methods of investigation[4]. Sedimentation analysis[3] and streaming birefringence[5] show the molecule to have an extended shape with a length of $2a = 890$ A and a minor axis $2b = 125$ to 150 A. The molecular weight is $8.9 \cdot 10^6$ calculated from sedimentation data. At certain changes in pH and ionic strength the molecule dissociates parallel with its longer axis so that the fractions have the same length as the original molecule. At low ionic strength and pH = 7, which were the conditions for this investigation, the molecule is undissociated and stable. Measurements on monolayer[6], which involves a rather harsh treatment of the molecules, gives a thickness corresponding to the dimension of the minor axis of a molecule dissociated into eights. Determinations of molecular dimensions from electron micrographs of hemocyanin have given various results which have been attributed to a decomposition on drying. However, POLSON AND WYCKOFF[7] have succeeded in getting well-defined pictures of Busycon hemocyanin which has properties very similar to those of Helix Pomatia hemocyanin. From their pictures it was concluded that electron micrographs confirm the correctness of the results obtained from sedimentation analysis. Therefore, in conclusion, it can be stated that different methods are in agreement and indicate that Helix Pomatia hemocyanin is a macromolecular compound with molecular dimensions as discussed above.

From the molecular dimensions the rotary diffusional constants and the relaxation times can be calculated for the hemocyanin molecule. The shortest possible calculated relaxation times are obtained when the smallest axial dimensions are used. Employing the values $2a = 890$ A and $2b = 125$ A gives the relaxation times in aqueous solution $\tau_a = 42 \cdot 10^{-6}$ sec. for rotation of the long axis and $\tau_b = 7.6 \cdot 10^{-6}$ sec. for rotation of the short axis[8]. It is of interest to compare these values with the relaxation time obtained from the dielectric dispersion. The lowest observed value for the critical frequency was 0.69 Mc. This value corresponds to a relaxation time of $0.22 \cdot 10^{-6}$ sec. Thus there is a discrepancy of a factor of 34 between the relaxation time calculated from known molecular dimensions and that obtained from dielectric data. This is far beyond the limits of experimental errors. If average values are used for molecular dimensions and dielectric dispersion frequencies, the discrepancies are even larger. For that reason it must be concluded that the dielectric dispersion cannot be caused by a relaxation phenomenon of a rotation of the whole hemocyanin molecule, nor can the dielectric increment be caused by a rotation of the macromolecules.

It was pointed out above that the hemocyanin molecule is built of and easily dissociates into eight subunits of the same length as the whole macromolecule. Though the experimental conditions always were such as to keep the hemocyanin molecule undissociated, the possibility should also be discussed here whether the dielectric properties could arise from rotations of these subunits. Though highly improbable, such a rotation of the small units might possibly occur within the large macromolecules when subjected to an electric field. If that is the case, the dielectric increment should be due to a rotation of the eights around their longest axis since that is the only type of movement that possibly could give a relaxation time not too far from that observed in the megacycle region. Further the subunits must be assumed to have a fixed dipole moment perpendicular to their longest axis. If the dielectric increment were due to such a rotation of the subunits with fixed dipole moments, either free in the solution or within the macromolecules, an increase in dielectric constant should be observed when the longer axes of the molecules were oriented perpendicular to the electric field (as opposed to random orientation) and a decrease when they were parallel to it. Though the velocity gradient ($G_c = 8{,}000$ sec^{-1}) used in our experiments was not capable of completely orienting all the hemocyanin molecules, the gradient was still sufficient to orient the molecules to an extent that the dielectric increment should change by more than 0.1 dielectric unit, which was the accuracy with which a streaming dielectric effect could be observed. This is evident from the fact that the parameter $\alpha = G/\theta_b = 8{,}000/11{,}900 = 0.67$ (G = velocity gradient and θ_b rotary diffusional constant for rotation about the small axis) corresponds to in the order of magnitude of 10% "completely" oriented molecules[8]. Since no increase in dielectric constant was ever observed when the electric field was perpendicular to the stream lines, and thus also perpendicular to the hydrodynamically oriented hemocyanin molecules, it must be inferred that the dielectric increment could not be caused by a rotation of the eight subunits of the macromolecule.

Therefore a new picture must be sought for the physical process behind the dielectric properties of macromolecular solutions like those of hemocyanin. Recently KIRKWOOD AND SHUMAKER[9] have proposed a new theory which indicates that the fluctuation of dipole moment due to mobile proton distribution could be of importance for the dielectric increment of macromolecular solutions. Whether their theory could explain the streaming dielectric results obtained cannot yet be definitively decided since the theory is not fully developed as far as the dielectric dispersion is concerned. It is interesting, however, that it is to be expected that the dielectric increment should be less sensitive to molecular orientations according to their mobile proton theory than according to the permanent dipole theory.

In the previous streaming dielectric investigations of sodium thymonucleate[1] and sodium hyaluronate[2], it was found that the state of the water structure of the solution is probably an important factor determining the dielectric properties. The hypothesis was suggested that the dielectric properties were mainly due to the quasi-crystalline order of an ice-like hydration shell surrounding the macromolecules[10]. It does not seem to be impossible that the dielectric properties of hemocyanin solutions are also mainly due to the hydration structure of the molecules rather than to the properties within the macromolecules themselves. This question will be further discussed elsewhere.

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SUMMARY

The dielectric dispersion and the dielectric increment have been measured for Helix Pomatia hemocyanin solutions subjected to velocity gradients in order to orient the molecules in relation to the electric field. No change in dielectric properties with the velocity gradient was observed when the electric field was perpendicular to the stream lines. The relaxation time obtained from dielectric dispersion frequencies disagrees by a factor of more than 30 from the relaxation time calculated from known molecular dimensions. It is concluded that the dielectric properties cannot arise from rotations of the whole macromolecules or of their eight subunits.

RÉSUMÉ

La dispersion ainsi que l'incrément diélectriques ont été mesurés pour des solutions d'hémocyanine (Helix Pomatia), celles-ci étant soumises à l'action d'un gradient de vitesse qui oriente les molécules par rapport au champs électrique. Les propriétés diélectriques ne changent pas quand le gradient de vitesse varie, le champs électrique étant perpendiculaire à la direction d'écoulement. Le temps de relaxation obtenu en partant de la fréquence de la dispersion diélectrique diffère du temps de relaxation calculé à partir des dimensions moléculaires connues d'un facteur de plus de 30. Nous en tirons la conclusion que les propriétés diélectriques ne résultent ni de la rotation des macro molécules entières ni de la rotation des huit unités dont chacune de ces macromolécules est constituée.

References p. 582.

ZUSAMMENFASSUNG

Die dielektrische Dispersion und das dielektrische Inkrement von Hämocyaninlösungen von Helix Pomatia wurden gemessen, unter gleichzeitiger Einwirkung eines Geschwindigkeitsgradienten, um die Molekeln im elektrischen Feld zu orientieren. Wir konnten keine Änderung der dielektrischen Eigenschaften mit varierendem Geschwindigkeitsgradient nachweisen, wenn das elektrische Feld senkrecht zur Strömungsrichtung lag. Die Relaxationszeit, die sich aus der Frequenz der dielektrischen Dispersion ergab, unterschied sich von der Relaxationszeit, die nach bekannten Moleküldimensionen berechnet wurde, um einen Faktor von mehr als 30. Daraus wurde geschlossen, dass die dielektrischen Eigenschaften nicht durch die Rotation der ganzen Makromoleküle oder ihrer ein Achtel grossen Unteneinheiten bedingt sein können.

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