of an internal rotation; the greater time for the carbon compound indicates some frictional resistance to rotation. Both dimethoxy compounds have similar relaxation times, as also have the tetramethoxy compounds. The greater size and substantially higher dipole moments of the silicon compounds, however, should produce a proportionately greater contribution from the molecular tumbling motion. The relaxation time for end-over-end rotation is approximately proportional to the molar volume and, when several relaxation mechanisms occur, the relative weighting of each depends upon the square of its orientating dipole moment.[15] Consequently, these results are only explicable if the internal dielectric relaxation by rotation of the methoxy groups occurs more readily in the silicon compounds. With three methoxy groups the carbon and silicon compounds have similar dipole moments but the four and a half-fold difference between their τ values again demonstrates the greater freedom of rotation in the silicon compound.

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Ultrasonic Absorption Mechanisms in Aqueous Solutions of Bovine Hemoglobin[1]

by W. D. O'Brien, Jr., and F. Dunn*

Bioacoustics Research Laboratory, University of Illinois, Urbana, Illinois 61801 *(Received June 21, 1971)*

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In order to study further the principal loss mechanisms of ultrasonic energy in biological media, the absorption and velocity were determined in aqueous solutions of hemoglobin, largely at 10°, over the frequency range 1–50 MHz. A distribution of relaxation processes is necessary to characterize the absorption spectra. Interaction of the acoustic wave with the hydration layer of the macromolecule, and not direct interaction with macromolecular configuration, appears as a dominant mechanism. The ultrasonic absorption titration curves exhibit maxima around pH 2–4 and pH 11–13, in addition to possessing a broad peak in the pH range 5–9. The peaks in the absorption titration and the similar ones observed for bovine serum albumin (*J. Phys. Chem.*, **73**, 4256 (1969)), are attributed to the proton-transfer reaction occurring between particular amino acid side chain groups and the solvent. The broad peak is partially attributed to the proton transfer resulting from the imidazolium function of the histidine residue.

Introduction

Although ultrasonic spectroscopy has been available, in principle, for at least two decades, to date only a few biological macromolecules have been examined. Probably the most extensively studied biopolymer is the globular protein hemoglobin, the oxygen carrier in the red blood cells of vertebrates.[2-4] The earliest work of importance is that of Carstensen, *et al.*,[5] who investigated the ultrasonic absorption and velocity in the blood, plasma and solutions of albumin, and hemoglobin, and concluded that the acoustical properties of blood are largely determined by the protein concentration. In addition they showed that the absorption coefficient of hemoglobin is approximately the same as that of serum albumin within the frequency range 0.8–3 MHz in the temperature range 10–40°.

Within the neutral pH region, aqueous solutions of hemoglobin have now been examined over the extended frequency range 35–1000 MHz[2,3] and it has been shown that it is possible to approximate the entire spectrum with four appropriately selected discrete relaxation processes.

More recently other globular proteins have been examined. Kessler and Dunn[6] studied aqueous solutions of bovine serum albumin and attributed the ultrasonic absorption in the neutral pH region to solvent–solute interactions. Outside of the neutral pH range,

(1) (a) Portions of this work were extracted from the Thesis submitted by W. D. O'Brien, Jr., in partial fulfillment of the requirement for the Ph.D. degree in electrical engineering, University of Illinois. (b) A preliminary report of this work was presented at the 78th meeting of the Acoustical Society of America in San Diego, Calif., Nov 1969 [*J. Acoust. Soc. Amer.*, **47**, 98 (1970)].

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the absorption behavior was thought to correlate with conformation changes. Wada, *et al.*,[7] investigated the ultrasonic absorption of gelatin at 3 MHz as a function of pH which had a peak in the absorption spectrum around pH 4 and was interpreted to be a dissociation type reaction of the protein side chains. Also it has been reported that the absorption magnitude of gelatin solutions within the frequency range 0.7–10 MHz is approximately half that in hemoglobin and albumin solutions.[8] For each of these protein solutions the ultrasonic absorption spectra exhibit a distribution of relaxation times.

Two synthetic polyamino acids have been examined under varying environmental conditions with respect to their ultrasonic absorption. The primary mechanism proposed to explain the excess ultrasonic absorption in poly-L-glutamic acid solutions[9] is that of the solvent–solute interaction whereas Schwarz[10] attributes the excess absorption to the helix–coil transition. The examination by Wada, *et al.*,[7] revealed that at 50 kHz the absorption mechanism is that of helix–coil transition while at 3 MHz the absorption is attributed to side chain dissociation. It has been shown[11] that the observed ultrasonic absorption behavior in aqueous poly-L-lysine solutions can be associated with the helix–coil transition.

The carbohydrate, dextran, a linear α (1–6) anhydroglucose polysaccharide, assumes a random coil conformation in solution whereas most proteins exist as a compact, rigid molecule. The ultrasonic absorption spectra of dextran, in the frequency range 3 to 69 MHz, can be represented by a distribution of relaxation times.[12] The absorption magnitude is considerably less than that exhibited by proteins, and has been attributed to the proteins possessing a secondary and tertiary structure while dextran does not. In addition, the protein gelatin, which does not possess a tertiary structure, also exhibits a lesser absorption magnitude than those proteins with higher ordered structure. Thus the suggestion that the tertiary structure may be responsible for some of the excess ultrasonic absorption observed in protein solutions appears to have received attention.[13,14]

It is apparent from above that the mechanisms mainly responsible for the ultrasonic absorption are unsettled. The present study was undertaken to provide additional data from observations at extreme pH values.

Experimental Techniques

Two distinct systems, described in detail elsewhere,[15,16] were utilized. The high frequency system, an automated version of that of Pellam and Galt,[17] utilizes a transmitting and a receiving transducer and has a lower frequency limit of approximately 9 MHz because of the apparent attenuation due to diffraction effects. The upper frequency limit is well beyond the 50 MHz employed here. At the lower frequencies, a comparison method is used which minimizes the difficulties due to diffraction,[5,18] and is capable of measuring ultrasonic absorption and velocity over the frequency range 0.3–20 MHz. Both systems employ the standard pulse techniques. These measurement techniques assume the absorption behavior of the fluid under investigation to be exponential in nature, *viz.*

$$p = p_0 e^{-\alpha x} \tag{1}$$

where p_0 is the pressure amplitude at $x = 0$, x is the acoustic path length over which the acoustic pulse travels, and α is the amplitude absorption coefficient per unit length. Speed of sound measurements are accomplished by adding algebraically the received signal to a reference signal and recording the time required to change the acoustic path length by 100 wavelengths.[16] The temperature of the liquid under investigation was maintained to $\pm 0.05°$.

Two grades of methemoglobin (Hb), obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, were investigated, *viz.*, uncrystallized Bovine Hemoglobin (Hb-OX), control no. 3099, and Hemoglobin Bovine 2X Crystalline (Hb-2X), control no. 1480, 8647, and 8995. Singly deionized and distilled water, testing to at most 0.15 ppm impurities[19] was used throughout. The protein solutions were prepared by placing the proper amount on top of a measured volume of water and refrigerating until mixing was complete, usually 2–5 hr. The uncrystallized hemoglobin solution, which contained some red blood cell structures, was centrifuged at 20,000*g* for 2 hr to remove heavier particles. This supernatant, as well as other protein solutions, were filtered twice through type A glass fiber filters (Gellman Inst. Co., Ann Arbor, Mich.) to remove particles larger than 0.3 μ in diameter and stored at 7° until used, usually not more than a few hours. The weight concentrations of the acoustically measured solutions were determined, to an accuracy of better

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(19) Tested at the Illinois State Water Survey, Champaign, Ill.

than ±0.3% at room temperature, by evaporating 15 ml in a tared beaker over air until dry and placing in a vacuum desiccator for 24 hr.

The pH of the hemoglobin solution was altered by the addition of standard volumetric solutions of either 1.0 N HCl or KOH. The acid or base was introduced slowly, to minimize pH gradients, into the circulating fluid within the acoustic chamber in steps to produce changes ranging from 0.1 to 1.5 pH units. The pH readings were obtained to within a relative accuracy of ±0.01 pH unit with a Beckman Century SS pH Meter using a Beckman pH combination electrode (39013) which fitted directly into the acoustic chamber. The pH meter was standardized at pH 2.01, 4.01, 7.00, 9.18, and 12.45.

In order to obtain the entire ultrasonic titration spectrogram, two measurement procedures were performed, each starting at neutral pH. A single procedure took from 8 to 20 hr, depending upon the number of points to be determined. Typically, the test liquid remained at a fixed pH for 1 hr, of which 15–20 min was allowed for the test liquid to equilibrate and the balance of time devoted to determining the ultrasonic absorption and velocity at the desired frequencies. Two complete titration spectrograms were obtained for each grade of hemoglobin in order to verify the results, the data being available in ref 15. The solutions, at the terminal pH values, were stored for several days at 7°, following the measurement procedure, with no observable precipitation occurring.

Results

The ultrasonic absorption of the two grades of hemoglobin was examined as a function of solute concentration to determine the onset of finite concentration effects. Deviation from linearity of Hb-OX solutions occurs around 0.10 g/cm^3 while that for the purer Hb-2X deviates around 0.16 g/cm^3, the latter agreeing with earlier reports.[20] Such deviation from linearity is commonly attributed to interactions among the solute molecules. Thus equating the volume of an assumed spherical particle and the volume per molecule, $4/3\pi R^3 = cN_A/M$, where M is the molecular weight of the biopolymer and N_A is Avogadro's number, yields a molecular radius R = 54 Å at the concentration, c, of 0.16 g/cm^3, *i.e.*, where the ultrasonic absorption begins to deviate from linearity. This value is in substantial agreement with that determined from X-ray diffraction techniques wherein the Hb molecule was found to be roughly spherical with overall dimensions of 64 Å by 55 Å by 50 Å.[21] In all the work reported herein, the concentration was maintained well below 0.16 g/cm^3.

The ultrasonic absorption data are presented in terms of the excess frequency-free absorption per unit concentration parameter

$$A = \Delta\alpha/cf^2 \qquad (2)$$

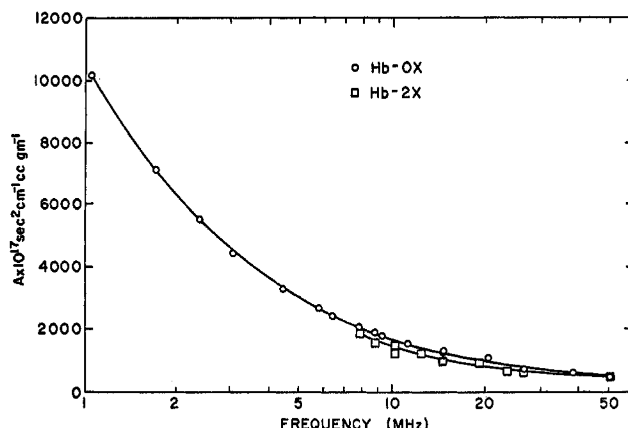

Figure 1. Ultrasonic absorption spectrogram in aqueous solutions of bovine hemoglobin (T = 10°).

where $\Delta\alpha$ is the difference between the absorption of the solution and that of the solvent, c is the biopolymer concentration in grams per cubic centimeter, and f is the ultrasonic frequency. The absorption parameter is shown in Figure 1 as a function of frequency for aqueous solutions of Hb-OX and Hb-2X at their isoelectric point, at 10.0°. The excess absorption exhibited by the uncrystallized Hb over the purer grade (about 10%) possibly reflects impurities contained in the former, and not present in the latter, which may also possess relaxational behavior. A similar situation has been reported for bovine serum albumin.[22]

The composite ultrasonic absorption titration curve is shown in Figure 2 for the frequency range 8.9–50.5 MHz and over the pH range 1.5–12.3. Similar shaped curves were also determined for Hb-OX down to 2.4 MHz.[15] The titration curves are similar in shape and magnitude to those for aqueous solutions for bovine serum albumin,[6] where the increase in A below pH 4.3 was associated with the intermediate N–F′ transition of Foster.[23] Hemoglobin, however, does not exhibit this transition. Similarly, in the alkaline pH region, both BSA and Hb show excess absorption peaking beyond pH 10.5.

The ultrasonic absorption coefficient and velocity were determined as functions of temperature over the range 10–37° in a neutral aqueous solution of Hb-2X at a concentration of 0.0349 g/cm^3 in order to provide information on apparent activation energies, shown in Table I with that of water. In all cases ΔF is less than that of water though it increases with increasing frequency. A similar experiment reported for BSA[6] indicates no frequency dependence within the neutral

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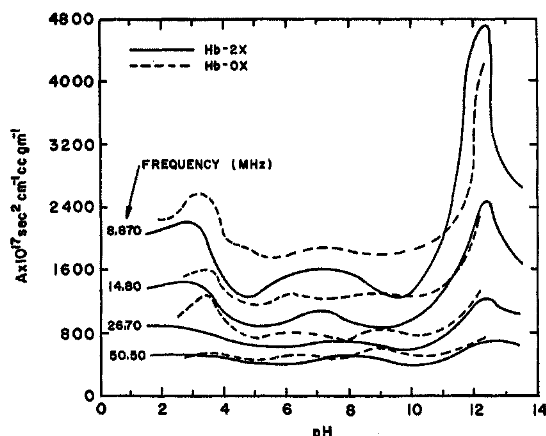

Figure 2. Composite ultrasonic absorption titration curve in aqueous solutions of bovine hemoglobin ($T = 10°$).

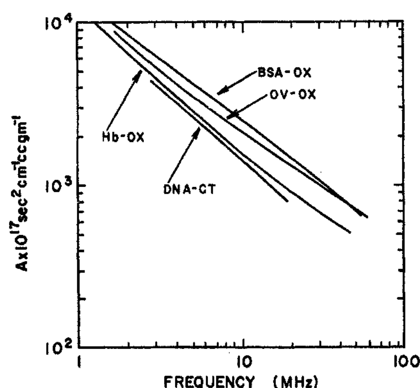

Figure 3. Composite ultrasonic absorption spectrogram, 10°: Hb-OX, uncrystallized bovine hemoglobin; BSA-OX, uncrystallized bovine serum albumin; OV-OX, uncrystallized ovalbumin; DNA-CT, calf thymus deoxyribose nucleic acid.

pH region and strong frequency dependence at pH 2.9, which was attributed to additional relaxation processes occurring in the acidic region. Such processess, resulting in strong frequency dependence of the activation energy, could be proton-transfer reactions occurring at neutral pH values in Hb and at pH 2.9 in BSA aqueous solutions which are absent in neutral pH BSA solutions.

Discussion

Figure 3 shows the absorption parameter A for aqueous solutions of Hb-OX, BSA, ovalbumin-uncrystallized,[24] and calf thymus DNA[25] at 10°. All four curves possess approximately the same frequency dependence although differences in magnitude are apparent. The former suggests that the mechanism(s) of ultrasonic absorption, for these four solutions, at their isoelectric point, may be the same. One universal feature of aqueous solutions of globular proteins and nucleic acids is the existence of the ubiquitous structuring of water about the macromolecule. It thus seems reasonable to speculate that the magnitude differences result from differing degrees of hydration, depending upon detailed molecular differences. Interaction of the solvent and solute has already been invoked to describe the excess ultrasonic absorption in aqueous solutions of biological molecules.[6,9] The present consideration is that the mechanism is the perturbation of the hydration layer, which is essential for maintaining the integrity of the biopolymer, by the acoustic wave process.

Hemoglobin is a globular protein composed of four subunits, two α polypeptide chains and two β polypeptide chains, with each consisting of approximately 144 amino acid residues and conjugated to a heme moiety (an iron-containing porphyrin derivative). No disulfide cross-links exist which would prevent each of the polypeptide chains from assuming a completely extended or helical configuration and each chain possesses approximately 65% helix content.[26] Under normal conditions approximately 6% of the human hemoglobin molecules in aqueous solution (pH 7; no denaturing agents present) are dissociated in half without loss of tertiary structure of the individual polypeptide chains.[27] This indicates the unique nature of the hemoglobin molecule to dissociate into its half subunits under very mild conditions without hydrolysis or pigment denaturation occurring. The nature of the bonds, or noncovalent links, which connect the individual polypeptide chains is unknown although it is assumed that the contacts are between α and β chains resulting in $\alpha\beta$ half molecules upon dissociation rather than α and β half molecules.[28] All forms of hemoglobin undergo major conformational changes when exposed to low pH. As the pH is lowered from the isoelectric point to 4.5, the hemoglobin molecule dissociates in half without appreciable change in the conformation of the resulting $\alpha\beta$ polypeptide chains.[29] As the pH is lowered from 7 to 3.5 to 2.9, the intrinsic viscosity increases from 3.5 cm^3/g[30] to 13.55 cm^3/g[30] to 17 $cm^3/$

Table I: Apparent Activation Energy of Aqueous Solution of Hb-2X (Concentration = 0.0349 g/cm³)

pH	f, MHz	ΔF, kcal/mol
6.9	8.870	1.2
6.9	14.80	2.0
6.9	26.70	2.6
6.9	50.50	3.4
Water	All	4.48

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g,[31] respectively. Thus at pH values less than 4, the Hb molecule clearly shows a marked expansion. Within the pH range 4.0–2.2, Polet and Steinhardt[32] reported that the heme is expelled from the apoprotein and the globin is unfolded, but the specific pH at which these two events occur is still unknown. Finally, Reichmann and Colvin[33] determined that around pH 2.0–1.8, the Hb molecule splits into four fragments of nearly equal size.

The hemoglobin molecule also dissociates at alkaline pH, *viz.*, the sedimentation coefficient decreases above pH 10, where it is 4.2×10^{-13} sec, reaching a value of 2.55×10^{-13} sec at pH 11.[34] Within the pH range 7–11, the diffusion coefficient remains relatively constant, indicating that the hemoglobin molecule dissociates in half as the pH is increased from 10 to 11, while the individual polypeptide chains retain their tertiary structure. As the pH is increased from 11, the sedimentation coefficient, along with the diffusion coefficient, decreases showing that the two subunits are expanding but not necessarily dissociating further.

Repulsion of like charges has been discounted as a mechanism of dissociation since at pH 6 the molecule possesses a charge of +5 whereas at pH 10 it is −30.[34] Thus the denaturation of Hb must be attributed to different mechanisms in the acidic and alkaline pH regions.

The effect of pH on the ultrasonic absorption characteristics of a number of aqueous solutions of amino acids have been investigated, *viz.*, serine and threonine,[35] glycine,[36,37] glutamic acid, aspartic acid and alanine,[36] and arginine and lysine,[38] and absorption maxima were observed in the acidic and alkaline pH regions. This absorption peaking, as a function of pH, has been described quantitatively for the above amino acids, assuming the proton-transfer reaction dominates the absorption process. However, the task of dealing with the similar peaking for macromolecules such as proteins which contain a large number of side chains able to participate in such reactions is far more difficult since detail of reaction coupling among sub-chain groups is not currently available. Thus a more qualitative discussion of the proton-transfer reaction for proteins must suffice for the present. For this reaction, which is a chemical relaxation mechanism, it is considered that the propagating acoustic wave disturbance perturbs the proton from the solvent (water) to the solute, an amino acid side chain, and *vice versa*. The energy necessary to drive the reaction is extracted from the acoustic field. The pH values at which the peaks in Figure 2 are maximum can be correlated with the pK values of the individual amino acid side chains which participate, both in the acidic and alkaline pH regions, *viz.*

$$\mathrm{pH_{acid}} = {}^1/_2(\mathrm{p}K + \mathrm{p}C) \quad (3)$$

and

$$\mathrm{pH_{base}} = {}^1/_2(14 + \mathrm{p}K - \mathrm{p}C) \quad (4)$$

where C is the molar concentration of the particular side chain group. Table II lists the amino acid side chains for Hb, along with their quantity in moles per 100,000 g of protein which can participate in the proton transfer reaction. Within the acidic pH range, the μ-carboxyl group (pK range on protein of 3.0–4.7) of aspartic acid (ASP) and glutamic acid (GLU) is primarily responsible for absorption peak. At the alkaline pH values, the ϵ-amino group (pK range 9.4–10.6) of lysine (LYS), the guanidinium group (pK range 9.8–10.4) of tyrosine (TYR), and the sulfhydryl group (pK range 9.4–10.8) of cysteine (CYS) contribute primarily to the absorption maximum. The diffuse peak, in Figure 2, occurs within the same pH range (5–9) in which about one-third, or 22, of the histidine groups (HIS) per Hb molecule titrate[39] suggesting that the broad peak around neutral pH may be a result of the imidazolium group (pK range 5.6–7.0), although the majority of the excess absorption still results from the

Table II: Proton-Transfer Side-Chain Groups (in mol/100,000 g of Protein)

Amino acid side chain	Human Hb[a]	Bovine Hb[b]	BSA[c]
ASP/GLU (ω-carboxyl)	162	194	152
LYS (ϵ-amino)	133	133	88
HIS (Imidazolium)	115	103	24.6
ARG (Guanidinium)	36.4	42.4	33.8
TYR (Phenolic hydroxyl)	36.4	36.4	29.3
CYS (Sulfhydryl)	18.2	6.1	0
α-Carboxyl	12.1	12.1	1.54
α-Amino	12.1	12.1	1.54

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interaction of the hydration layer with the acoustic wave.

Bovine serum albumin consists of a single polypeptide chain with molecular weight of about 68,000. The conformation of the molecule remains unchanged at pH values between 4.3 and 10.5 and sedimentation coefficients decrease, with their ratios remaining constant,[40] implying that the molecule expands but does not dissociate. The intrinsic viscosity of the BSA molecule expands in steps, from 3.6 to 4.5 cm^3/g around pH 4 and from 4.5 to 8.4 cm^3/g around pH 3.[41] At $[\eta] = 8.4$ cm^3/g, the BSA molecule is not highly expanded, and this can be attributed to the large number of disulfide bonds the molecule possesses. Tanford, *et al.*,[41] and Weber[42] have speculated that the alkaline expansion of the BSA molecule is similar to that in the acid pH region but this has neither been confirmed nor denied[23] by experiment.

The effect of the protonation reaction of the imidazolium group of the histidine amino acid to the results of serum albumin[6] appears to be minimal on the ultrasonic absorption within the pH range 5–9, possibly because of the small number of histidine amino acids in serum albumin (Table II). For both Hb and BSA, there is approximately the same ratio of amino acid groups which can partake in the proton transfer reaction between the alkaline (LYS, ARG, TYR, CYS) and acidic (ASP, GLU) pH region and as shown in Table II, these concentration ratios are 217/194 for Hb and 151/152 for BSA. The similarity of these ratios is considered to lend support to the view that the similar ultrasonic absorption magnitude of the peaks of these two globular proteins results from the same mechanism. Previously[6] the peaks in the BSA spectrogram were discussed in terms of conformational changes such as Foster's[23] N–F′ transition. It now appears that a more acceptable explanation for such peaks in the ultrasonic absorption titration curves of Hb and BSA may be the proton-transfer reaction. However, the role of conformation cannot be completely discounted at this time as the evidence for each mechanism (1) is less convincing than one would like and (2) does not exclude the other from being present, simultaneously, to some degree of effectiveness.

Acknowledgment. The authors acknowledge gratefully the support of this research by a grant from the Institute of General Medical Sciences, National Institutes of Health.

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