Ultrasonic absorption by solvent–solute interactions and proton transfer in aqueous solutions of peptides and small proteins

Philip R. Strom-Jensen[a] and Floyd Dunn
Bioacoustics Research Laboratory, University of Illinois, 1406 West Green Street, Urbana, Illinois 61801

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Ultrasonic absorption values in aqueous solutions of myoglobin, apomyoglobin, α-lactalbumin, bacitracin, and the C- and N-terminal CNBr cleavage fragments of myoglobin were determined by the cylindrical resonator technique at temperatures of 4 °C and 20 °C in the frequency range 0.5–7 MHz. A proposed mechanism of absorption in solutions of proteins and peptides is perturbation by the ultrasound of the equilibrium between protein bound and free water, producing a distribution of relaxation processes occurring within the frequency range of the investigation. The hypothesis is supported by the dependence of the ultrasonic absorption on solute surface area and molecular weight. Another mechanism of absorption is that due to proton transfer which occurs noticeably in the presence of phosphate ions. Those proteins (myoglobin and α-lactalbumin) with significant globular structure appear to undergo additional relaxation processes at frequencies above 7 MHz.

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INTRODUCTION

The widespread use of ultrasound in diagnostic and therapeutic medicine presents a need for a thorough understanding of the mechanisms of interaction of ultrasound and biological tissues. Numerous studies have been directed toward the bulk ultrasonic properties of intact tissues.[1,2] These, and others, reveal the importance of proteins in the determination of ultrasonic absorption.[3] In the case of blood specimens, for example, the absorption coefficient has been found to be proportional to the weight percent of the protein content, and this has encouraged considerable effort to be expended to understand the absorption by proteins and their analogs in solution.[4] Through study of amino acids,[4–7] polyamino acids,[8,9] and soluble proteins[10–13] in aqueous solution, several mechanisms have been proposed to account for the high value of ultrasonic absorption by proteins. One mechanism that has been shown to be involved in absorption at *p*H extremes is the relaxation of proton-transfer equilibria occurring at ionizable residues.[14] More recent measurements indicate that this mechanism may also be important under physiological conditions.[15] Other studies suggest that relaxation of solvent–solute equilibria may be important in determining ultrasonic absorption in protein solutions.[11,16] Other mechanisms have been proposed and it is difficult, at this stage, to select any single mechanism as predominant in causing ultrasonic absorption by proteins.[17]

The experiments undertaken in this investigation have been aimed at gaining a better understanding of the mechanisms of absorption by proteins under physiological conditions, and have involved proteins and peptides whose low molecular weights are in a range which has received less attention than the larger globular proteins. Similar studies with aqueous solutions of the polymers dextran and polyethylene glycol show an interesting dependence of ultrasonic absorption on molecular weight.[18–20]

Results from the protein and peptide solutions of this study lend support for an ultrasonic absorption mechanism involving perturbation of the hydration layer surrounding these molecules. Such a mechanism also appears to make a greater contribution in cases where the molecule exists more as a random coil than as a compact globular structure. Under the proper conditions, proton transfer between the solvent and ionizable groups on the solute molecule also makes a significant contribution to the total absorption. Specifically, around *p*H 7 such proton transfer occurs efficiently in the presence of phosphate ions.

I. MATERIALS AND METHODS

Sperm whale myoglobin, purchased from U.S. Biochemical Corp. (lots #9825 and #44121), was specified by the manufacturer to be salt-free and was used without further purification. It dissolved readily in water for direct ultrasonic measurement or biochemical treatment.

Apomyoglobin was produced in the laboratory by the acid methylethyl ketone method,[21] in which the heme group is removed to an organic phase and the apomyoglobin recovered after lyophilization of an aqueous phase.

Smaller peptides were produced from the apomyoglobin by cleaving at methionine residues using cyanogen bromide. The cleavage reaction was carried out at concentrations of apomyoglobin and cyanogen bromide of 20 and 8 mg/ml, respectively, in 70% formic acid. The reaction proceeded at room temperature for 24 h after which the excess cyanogen bromide was removed to a cold trap by rotary evaporation at 25 °C. A dry product was then obtained by lyophilization.

The method of isolation of the peptides was adapted from Marshall *et al.*[22] After lyophilization, the material was dissolved in 10% acetic acid at a concentration of 50 mg/ml. The sample was then applied in 10-ml quantities to a chromatography column (4×180 cm) of Sephadex G-50-fine (Pharmacia Fine Chemicals) equilibrated with 10% acetic acid and eluted with this solvent at a rate of 60 ml/h. The

[a]Current address: Miles Laboratories, Inc., P. O. Box 70, Elkhart, IN 46515.

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elution fractions were collected by a Gilson Micro Fractionator. The fractional positions of the peptides were identified by optical absorption at 276 nm.

Three main peaks were always observed and their identities verified by gel electrophoresis. The first peak contained unreacted myoglobin. The second and third peaks correspond to the N-terminal and C-terminal peptides, respectively, and are hereafter referred to as peptide P2 and peptide P3. Since myoglobin contains two methionine residues, another peptide, larger than P2 or P3 is expected. In some column runs, a small peak was observed before that of peptide P2, and contained the central myoglobin peptide. However, the recovery of this peptide was always too small to allow sufficient accumulation for ultrasonic study.

The milk protein α-lactalbumin used in this investigation was isolated from a crude lactalbumin product purchased from U.S. Biochemical Corp. (lot #19371). The crude lactalbumin is an acid precipitate from bovine milk and contains the protein α-lactalbumin as a minor component. The method of isolation was adapted from that of Robbins and Kronman.[23] A salt-free dry product was obtained by passage of an α-lactalbumin solution through a column of Sephadex G-10, followed by lyophilization. The recovered material produced a single band on SDS gel electrophoresis.

The antibiotic bacitracin was purchased from U.S. Biochemical Corp. (lot #26581) and used without further purification.

The protein and peptide solutions were prepared from the lyophilized chemicals in 10 ml of distilled water. The solution was then filtered, to remove any undissolved residue, using a Millex-HA syringe filter, pore size 0.45 μm (Millipore Corp., Bedford, MA). The solution was stored at 5 °C in a flask overnight. Immediately prior to ultrasonic measurements, the solution was degassed for 10–15 min under a reduced pressure (about 5 mm Hg) in a rotary evaporator to eliminate microbubbles which could interfere with the sound wave propagation. Concentrations of the specimen solutions were determined after the ultrasonic measurements by determining the dry weight of two 3-ml aliquots after lyophilization. Concentrations were always between 1% and 2%, a range in which the absorption was virtually linear with concentration.

Acoustic absorption measurements were made using a cylindrical resonator after the design of Labhardt and Schwarz.[24] Figure 1 is a cross-sectional view of the resonator which is a figure of revolution about the axis A–A'. The 1-in.-diam, X-cut quartz transducers (Valpey–Fisher Corp., Hopkinton, MA) were supplied with overtone-polish surfaces and gold-on-chromium electrodes. They were "wrung-on" to the recess in the frame with a thin layer of high-vacuum silicone grease (Dow–Corning). An elevated hydrostatic pressure could be maintained in the specimen cavity by application of regulated compressed air to one of the ports. Such an overpressure presumably reduces the effects of diffraction by producing a slight concavity of the quartz transducers and thereby extends the usable lower frequency range.[25] An increased pressure of 12 psi (83 kPa) extended the low-frequency limit to 0.4 MHz, from a 1-MHz limit at ambient pressure.

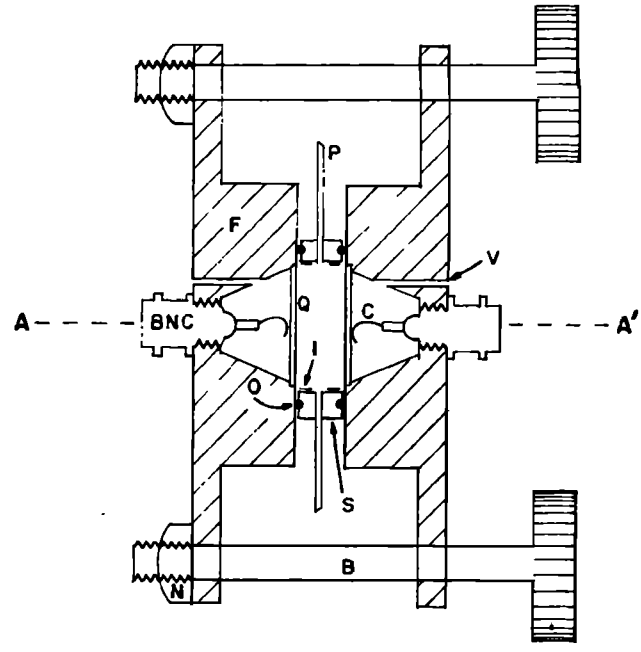

FIG. 1. Cross-sectional view of cylindrical resonator. F, stainless steel frame; Q, quartz crystal transducers; S, Plexiglas spacer; O, rubber O-ring; P, filling port; I, rubber inlay; V, vent hole; C, electrode contact; B, adjusting bolt.

Temperature control was provided by a Neslab Exacal 500 constant temperature bath and an accompanying Endocal 350 refrigeration unit (Neslab, Portsmouth, NH). The bath, with a volume of 45 l, was filled with a mixture of distilled water and ethylene glycol, 50:50 by volume, and maintained the temperature constant to less than 0.01 °C during the 5–10 h necessary to accomplish a set of absorption measurements.

A synthesized signal generator (Hewlett–Packard 8660B) provided the voltage for the transmitting transducer and displayed the driving frequency to the nearest hertz. The output voltage from the receiving transducer was normally connected directly to a spectrum analyzer (Hewlett–Packard 8552A and 8553B) which served as a tuned receiver yielding the amplitude of the received signal. When the output voltage was too low to be displayed adequately by the spectrum analyzer alone, a wideband preamplifier (Hewlett–Packard 461A) was inserted between the resonance cell and the analyzer.

From the 3-dB bandwidth of a longitudinal resonance, the excess pressure absorption coefficient, α_{ex}, due to the solute can be determined according to[24]

$$\alpha_{ex}\lambda = \pi(\Delta f_S - \Delta f_R)/f_0, \tag{1}$$

where Δf_S and Δf_R are the 3-dB bandwidths of the resonator containing specimen and reference liquid, respectively, and f_0 is the center frequency of the resonance. The reference liquid was a NaCl solution whose concentration was chosen to match, with an accuracy of $\pm$ 1 m/s, the velocity of sound in the specimen solution. At the NaCl concentrations used, these reference solutions have a negligible absorption difference from distilled water, in comparison with the large absorption of the specimen solutions.

The absorption data are presented as the specific frequency-free absorption coefficients A defined as

$$A = \alpha_{ex}/f^2C, \quad (2)$$

where f is the frequency of measurement and C is the concentration of solute in grams per milliliter. The absorption values obtained by the resonator method in this study are estimated to be accurate to within $\pm$ 5%, based on comparison measurements with aqueous solutions of bovine serum albumin.[16]

II. RESULTS

Absorption measurements were made on the proteins and peptides listed in Table I. Initial measurements were made in unbuffered aqueous solutions at *p*H 7.0, with the exception of apomyoglobin and peptide P2 which, due to their insolubility at that *p*H, were measured at *p*H 5.2. All measurements were carried out at 20.0 °C unless otherwise specified.

Figures 2 and 3 show the frequency dependence of the specific frequency-free absorption coefficient for the six compounds (the data have been separated into two graphs for ease of readability). The general appearance of these data is similar to that for other soluble proteins such as hemoglobin.[11,13] In the latter case, the behavior of the absorption in this frequency range has been deduced to be the result of more than one relaxation process.

Figure 4 shows the absorption parameter A plotted against the molecular weight of the solute. It is to be noted that at a given frequency, data for four of the molecules fall along smooth curves. The values of the absorption parameter A of myoglobin and α-lactalbumin, however, are consistently below those of the other compounds. Also included in this figure, for sake of qualitative comparison, are values of A, at 10 MHz, for solutions of diglycine and triglycine from another study.[4] In general, for all the compounds, A increases with molecular weight and this phenomenon is more pronounced at lower frequencies.

The effect of phosphate ions on the absorption in solutions of myoglobin and peptide P3 was studied at 4.0 °C and *p*H 7.0 and the results of these measurements are shown in Figs. 5 and 6. In both cases the effect of the phosphate ion is to increase the absorption compared to that when distilled water alone is the solvent. The algebraic differences in absorption for these polypeptides, with and without phosphate present, are shown in Fig. 7. Also shown in Fig. 7 are solid curves representing the theoretical single relaxation process

$$\alpha/f^2 = \{D_\tau/[1 + (f/f_r)^2]\} + B, \quad (3)$$

where f_r is the relaxation frequency, τ is the relaxation time, and D and B are constants.

TABLE I. Polypeptides studied in aqueous solutions.

Material	Molecular weight
Myoglobin	16 900
Apomyoglobin	16 280
α-Lactalbumin	14 000
Peptide P2	6320
Peptide P3	2570
Bacitracin	1420

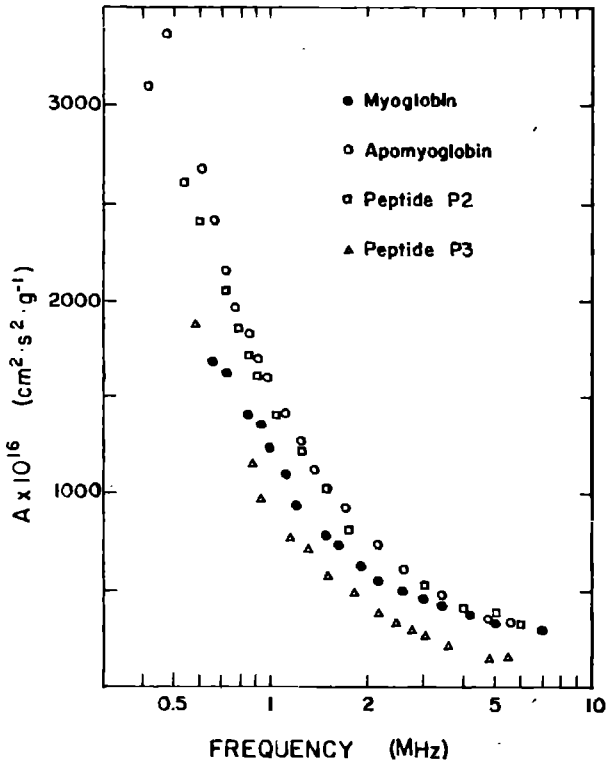

FIG. 2. Specific frequency-free ultrasonic absorption versus frequency for solutions of myoglobin, apomyoglobin, peptide P2, and peptide P3 at 20 °C.

III. DISCUSSION

The dependence of the absorption parameter A on molecular weight shown in Fig. 4 bears a great resemblance to similar dependencies for the polymers dextran[19] and polyethylene glycol.[20] In the case of dextran there is strong evidence to support the theory that the mechanism responsible for this behavior is an interaction between the sound wave and the structure of solvating water molecules around the polymer.[18] The theory describing this interaction involves perturbation of the equilibrium between water bound in a

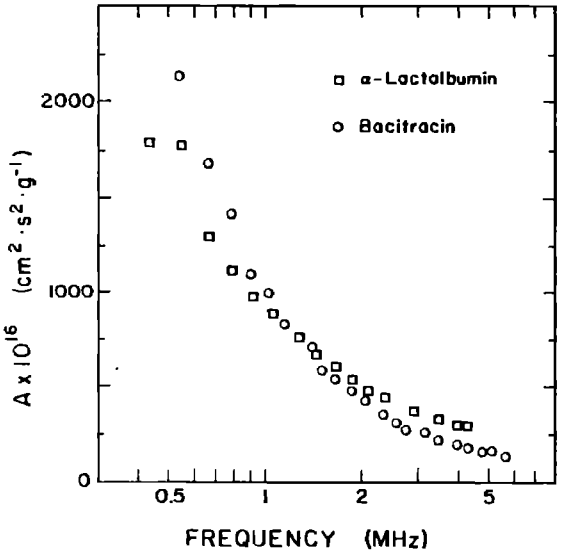

FIG. 3. Specific frequency-free ultrasonic absorption versus frequency for solutions of α-lactalbumin and bacitracin at 20 °C.

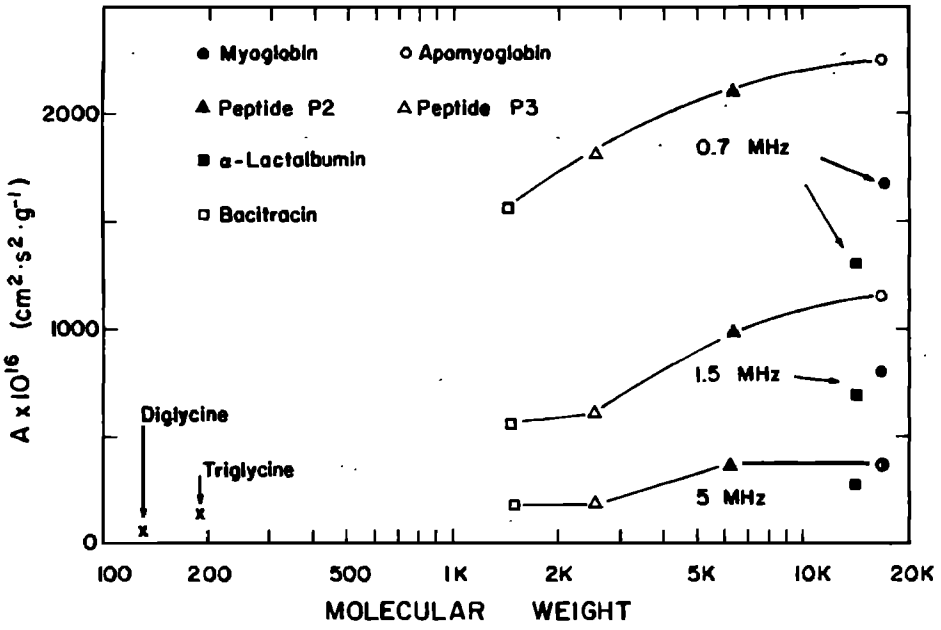

FIG. 4. Specific frequency-free ultrasonic absorption versus molecular weight at frequencies of 0.7, 1.5, and 5 MHz for solutions of myoglobin, apomyoglobin, peptide P2, peptide P3, α-lactalbumin, and bacitracin. Values also shown for diglycine and triglycine at 10 MHz.[4]

structure around the polymer and water free in the bulk solvent by the ultrasound, resulting in relaxation phenomena. The actual effect is probably through a perturbation, by the sound wave, of the segmental motion of the polymer chain in such a way that rearrangement of the water molecules is produced which interacts weakly with the chain. Such a mechanism has also been mentioned in analysis of the absorption characteristics of polyethylene glycol.[20,26]

Based on comparison with the nonprotein polymers discussed above, it is suggested that for those molecules whose A values fall along the smooth curves of Fig. 4, some part of the ultrasonic absorption is due to perturbation of the hydration equilibrium. As in the case of dextran, this may be coupled to the ultrasound through motions of the peptide chain itself.

The fact that A values for myoglobin and α-lactalbumin fall below those of the other specimens may be due to their extensive helical secondary and globular tertiary structure, and can be partially explained by considering the exposed surface areas of these molecules. If the simplifying assumption is made that the polypeptides exist either as globular proteins or as unfolded chains, then it is permissible to use the following empirical formulas, given by Richards,[27] to compute the surface areas exposed

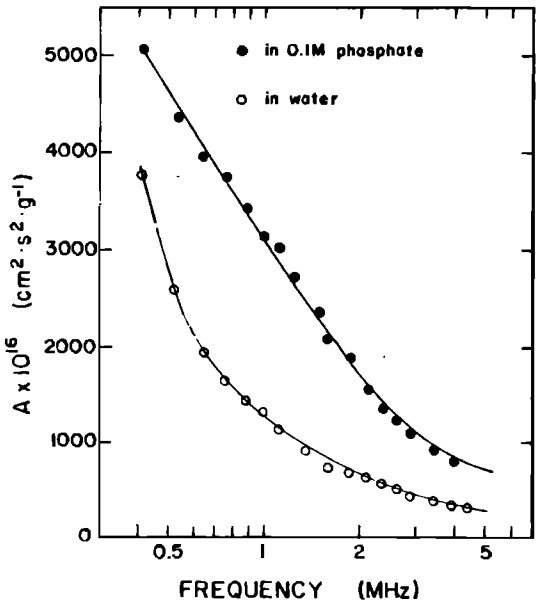

FIG. 5. The effect of phosphate ions on the specific frequency-free ultrasonic absorption in myoglobin solutions at pH 7.0 and 4 °C. Drawn curves have no theoretical significance.

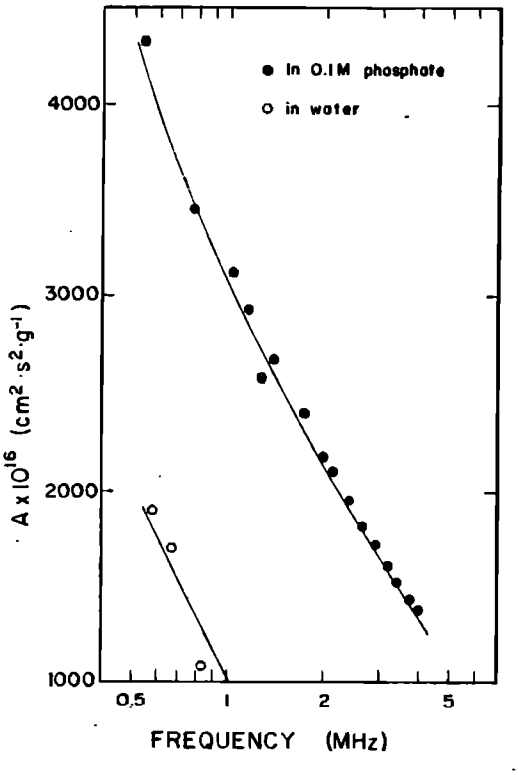

FIG. 6. The effect of phosphate ions on the specific frequency-free ultrasonic absorption in peptide P3 solutions at pH 7.0 and 4 °C. Drawn curves have no theoretical significance.

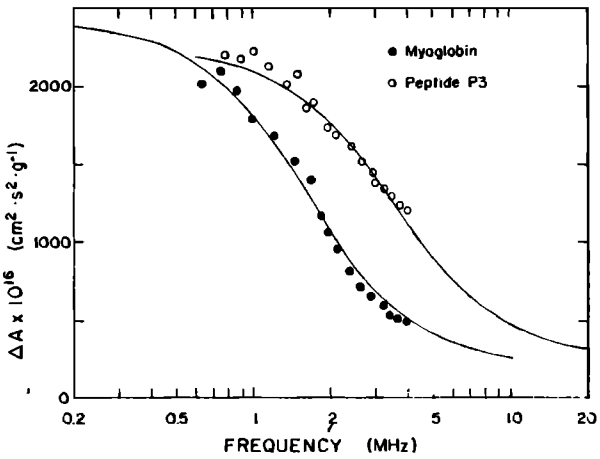

FIG. 7. Phosphate induced absorption versus frequency. Algebraic difference between specific frequency-free absorption with and without phosphate ions present is shown for myoglobin and peptide P3. The solid curves are single relaxation curves of Eq. (3) using the parameters $f_r = 1.65$ MHz, $B/C = 200\times10^{-16}$ cm^2 s^2 g^{-1}, $D\tau/C = 2220\times10^{-16}$ cm^2 s^2 g^{-1} for myoglobin and $f_r = 3.5$ MHz, $B/C = 250\times10^{-16}$ cm^2 s^2 g^{-1}, $D\tau/C = 2000\times10^{-16}$ cm^2 s^2 g^{-1} for peptide P3.

$$A_G = 11.12\,M^{2/3}, \tag{4}$$

$$A_U = 1.45\,M, \tag{5}$$

where A_G and A_U are the areas, in Å^2, of globular and unfolded proteins, respectively, and M is the molecular weight. Using Eq. (4), the surfaces areas of α-lactalbumin and myoglobin are 6459 Å^2 and 7323 Å^2, respectively. Next, using Eq. (5), we find that the equivalent unfolded molecular weights for these proteins, retaining the same exposed surface area, would be 4455 for α-lactalbumin and 5050 for myoglobin. Using the data from Fig. 4 at 1.5 MHz as an example, it is seen that, based on their ultrasonic absorption, α-lactalbumin and myoglobin behave in this respect similarly to unfolded peptides with molecular weights of approximately 3000 and 4000, respectively. Thus the actual ultrasonic absorption for these two globular proteins is still less than would be predicted based on the consideration of surface area.

Another feature important in determining solvent–solute interactions is the chemical nature (polar, charged, nonpolar) of the exposed surface. Since globular proteins generally "bury" a disproportionate number of nonpolar residues in their hydrophobic interiors, it is possible that the exposed residues of the other four polypeptides bestow on them a different overall chemical nature which is reflected in a slightly different ultrasonic absorption. Quantitative evaluation of this effect is not possible due to an incomplete knowledge of the secondary and tertiary structures of all the peptides. It is, however, possible to suggest that a combination of the magnitude and of the chemical nature of the surfaces of myoglobin and α-lactalbumin cause their absorption values to appear below those of the other specimens.

Slutsky *et al.*[15] have examined the effect of phosphate buffered solution on the ultrasonic absorption by bacitracin. The pK of the single histidine residue of bacitracin is such that significant proton exchange may occur between this moeity and phosphate ion around neutral pH and temperatures around 4 °C. The difference between the absorption by bacitracin in the presence and in the absence of phosphate ion was, in fact, well fit by a single relaxation process centered at 1.2 MHz. When modeled as proton transfer relaxation, kinetic and thermodynamic parameters were obtained in good agreement with theoretical predictions. Such a mechanism was proposed by these investigators as a significant contributor to *in vitro* absorption in tissues.

The behavior due to phosphate observed in myoglobin solutions (Fig. 7) may be due to proton transfer between the phosphate ions and residues (presumably histidines) of the protein with pK values near 7. A single relaxation curve is employed to fit the data for myoglobin. Despite the good fit by this theory, the limited frequency range of the data prevents conclusive proof of single relaxation behavior. The presence of more than one relaxing element would not be surprising since myoglobin, as a complex protein, has more than one site at which proton transfer might occur under these circumstances. However, the nearness of fit suggests that any multiple relaxation processes of comparable magnitude in the investigated range are centered near the same frequency. In addition, the relaxation frequency of 1.65 MHz is close to the value of 1.2 MHz obtained by Slutsky *et al.*[15] for the histidine residue of bacitracin.

Solutions of peptide P3 exhibit, qualitatively, the same absorption behavior in the presence of phosphate ions as do solutions of myoglobin (Fig. 7). Although peptide P3 contains no histidine residues, proton transfer may still occur between the terminal amino group and the phosphate ions in solution. Again, a single relaxation curve is shown for the purpose of comparison to the data for this peptide. As with myoglobin, the frequency range of the data does not allow verification of only a single relaxation process. The presence of multiple relaxation processes or a continuous distribution of relaxation processes with a mean relaxation frequency of 3.5 MHz is also possible. The parameters used to generate this curve are consistent with a mechanism of proton transfer, which, in the case of peptide P3, occurs at a faster rate than in myoglobin as evidenced by the relaxation frequency of 3.5 MHz. This is not surprising since the amino group involved in proton exchange is at the end of the peptide chain and therefore less subject to stearic hindrance from adjacent residues. Such an interpretation of the data for peptide P3, as well as that for myoglobin, is in accord with the findings of other investigators.[15,28]

Returning to the frequency dependence of the ultrasonic absorption, it is considered that such behavior is most likely the result of a distribution of relaxation processes, some of which are structural relaxations of the solvent surrounding the peptide chains. If the data from Figs. 2 and 3 are replotted in a double logarithmic form, as in Figs. 8 and 9, an interesting property is revealed. Notice that while the curves for myoglobin and α-lactalbumin show a definite tendency toward leveling off at the higher frequencies, such is not the case for the curves representing the peptides. A double logarithmic plot of frequency-free absorption against frequency will not level off at high frequency in the absence of a relaxation process occurring at a still higher frequency. The straight line curves for bacitracin and peptides P2 and P3 in

Figs. 8 and 9 indicate that no relaxation processes are associated with these specimens at higher frequencies, or that if any such relaxation processes exist, their ultrasonic absorption magnitudes are much smaller than the magnitudes of those occurring in the frequency range of this investigation. The data for myoglobin and α-lactalbumin, however, show a definite leveling off, indicating the presence of significant relaxational absorption at higher frequencies.

In general, these higher-frequency relaxation processes may be related to the degree of structural complexity in the molecule. Optical measurements by Hermans and Puett[29] show that the peptides P2 and P3 contain 15% and 8% helical structure, respectively, under the conditions present during these measurements. Myoglobin, on the other hand, consists of a great deal more helical structure and a complex tertiary structure. Like myoglobin, α-lactalbumin is a complex structure (very similar to lysozyme). Thus the higher-order structure present in myoglobin and α-lactalbumin seems to be associated with a significant relaxation at frequencies higher than those employed in this study. The lack of such structure in bacitracin and the peptides P2 and P3 may account for the lack of significant higher-frequency contribution to the relaxation spectrum of these materials.

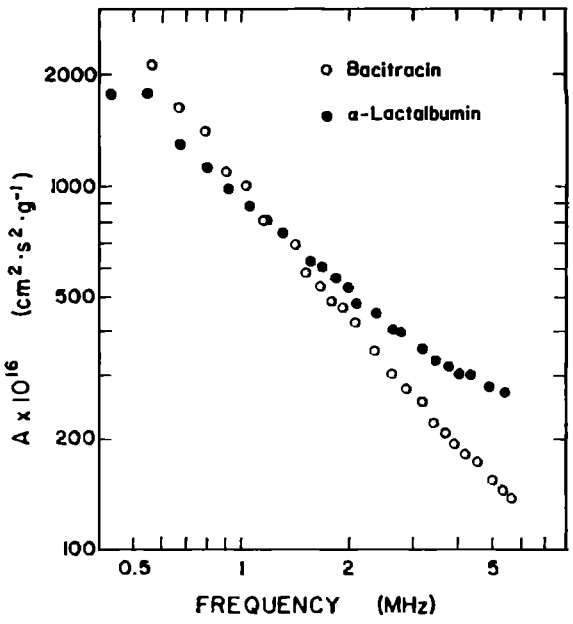

FIG. 9. Specific frequency-free ultrasonic absorption versus frequency for bacitracin and α-lactalbumin.

IV. CONCLUDING REMARKS

Results presented above indicate that perturbation of solvent structure around proteins and peptides makes a contribution to the absorption of ultrasound. This hypothesis is supported by the molecular weight dependence (Fig. 4) of the absorption. The solvent-related absorption makes a greater contribution to the absorption of proteins and peptides with significant random coil structure than it does to proteins which are predominantly globular. This may be due to the smaller surface area per unit weight of the globular molecules or to a different chemical nature (amino acids) of the surface.

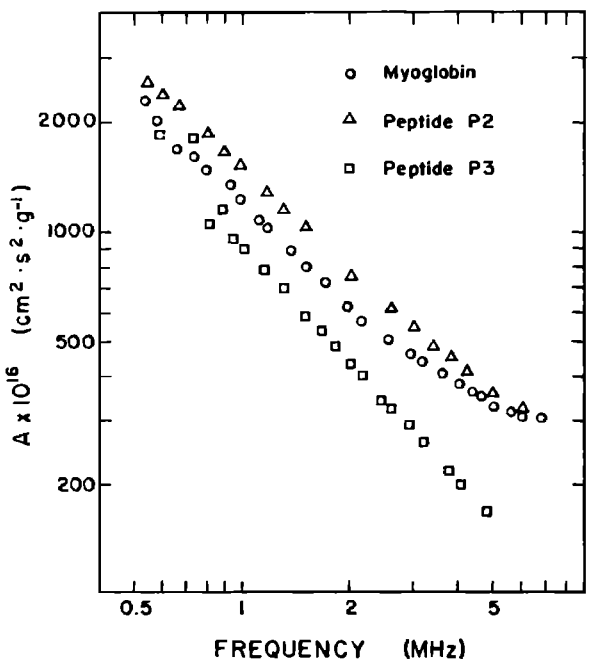

FIG. 8. Specific frequency-free ultrasonic absorption versus frequency for myoglobin, peptide P2, and peptide P3.

The molecular weight dependence of the absorption may be related to a cooperative phenomenon occurring in the solvent. Such a mechanism has been proposed by Hammes and Schimmel[26] to account for the molecular weight dependence of relaxation times observed in solutions of polyethylene glycol. As the size of the polymers increases, so may the number of water molecules which participate as a unit in the equilibrium between free and bound water. As the size of the cooperative unit increases, so will the absorption associated with this equilibrium, until an upper limit on the size of the cooperative unit is reached. Such an upper limit may be imposed by the period of the wave as compared to the time required to "communicate" a cooperative change among neighboring water molecules. This would account for the plateau in absorption at the high molecular weights characteristic of the polymers discussed above. A cooperative model also provides an explanation for the frequency dependence of the molecular weight effect seen in Fig. 4. If the maximum cooperative unit size is directly related to the period of the ultrasonic wave, then a smaller maximum size would be expected at higher frequencies. This is indeed the case for the data presented in Fig. 4.

In addition to solvent–structure mechanisms other mechanisms also play an important role in determining the overall absorption by proteins. The present results of myoglobin and peptide P3 in phosphate solution and the calculations of Slutsky *et al.*[15,28] show that proton exchange with phosphate ions accounts for a large part of absorption in soft tissues. Also, internal motions of side chains, as suggested by Kanda *et al.*[17] probably are, in part, responsible for the absorption characteristics of globular proteins. A complete model for absorption by proteins would include, at least, all of these mechanisms.

ACKNOWLEDGMENTS

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