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Ultrasonic Investigation of the Conformal Changes of Bovine Serum Albumin in Aqueous Solution

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The excess ultrasonic absorption and the speed of sound were measured in aqueous solutions of bovine serum albumin (a globular protein which undergoes marked configurational change with pH) at 20° over the frequency range 0.3 to 163 MHz and over the pH range 2.3 to 11.8. A sharp increase in the excess absorption is found outside the range 4.3 < pH < 10.5. The effect is reversible throughout this range and is more pronounced at the lower frequencies. The increase in the absorption below pH 4.3 appears to be correlated with the intermediate N-F' transition discussed by Foster and the change above pH 10.5 is thought to correspond to expansion of the molecule. At neutral pH, the ultrasonic absorption spectrum and the velocity dispersion are indicative of a broad distribution of relaxation processes. The magnitude of the ultrasonic absorption over the range 4.3 < pH < 10.5 is attributed to the perturbation of the solute-solvent equilibrium by the sound wave. Based on data at 20°, between 2.4 and 50 MHz, the frequency spectrum of the absorption increase at pH 3.5 over that at pH 7.0 may be described by a single relaxation process whose characteristic frequency is 2.2 MHz. Based on measurements of the velocity of sound at pH 7.0, the bulk modulus of BSA has been found to be $3.86 \times 10^9$ n/m².

Introduction

The spatial configuration assumed by a macromolecule within the environment of solvent molecules plays an important role in determining the hydrodynamic properties of the solution, as well as the chemical activity of the solute molecule. It appears that in cellular processes, for example, the transport of molecules across the protoplasmic membrane is related, in some complex manner, to the spatial geometry of the transported molecule as well as to the spatial arrangement of the lipoprotein complex constituting the cell membrane. Thus, it is within the realm of profitable investigation to explore methods which have the potential of providing information about molecular configuration and changes in molecule structure. Ultrasonic techniques have already been employed successfully to observe the dynamic equilibrium between multiple isomeric forms of molecules and to arrive at more complete kinetic descriptions of chemical and structural reactions whose relaxation times are comparable to the period of the ultrasonic wave. The adiabatic propagation of a longitudinal acoustic wave through a fluid medium results in time-varying, localized changes in pressure, density, and temperature. Thus, the wave motion may perturb molecular equilibrium at rates which depend upon the sound frequency. For a nonideal fluid, this leads to a time lag between an applied pressure and the ensuing change in density. Consequently, molecular energy level populations are perturbed at the expense of acoustic wave energy, and the process is referred to as absorption. Ultrasonic absorption spectroscopy is useful for studying fast reactions having rate constants over the range from $10^{-9}$ sec to $10^{-4}$ sec.

The reader unacquainted with ultrasonic technology may wish to consult ref 4 and 6 for details of theory and experimental methods for determining absorption and velocity, and for current reviews of measurements on liquids, including biological media.

Only a few biologically important macromolecules have been subjected to thorough ultrasonic examination. For both hemoglobin, a globular protein, and dextran, a random coil molecule, the interaction between solvent and solute is probably the principal mechanism of acoustic absorption. The pressure variation associated with the sound wave perturbs the equilibrium distribution of solvent molecules that are weakly bonded to the solute, and, since rearrangement

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(1) a Portions of this work were extracted from the Thesis submitted by the first-named author in partial fulfillment of the requirements for the Ph.D. degree in Electrical Engineering, University of Illinois, 1966. (b) Research Department, Zenith Radio Corporation, 6001 W. Dickens, Chicago, Ill. 60639.


of the solvent molecules does not occur instantaneously, absorption results. Of those biomacromolecules studied, hemoglobin can be made to undergo configurational changes, although ultrasonic spectroscopy has not been employed to study them. Polyglutamic acid, a synthetic polypeptide that can undergo a configurational change from helix coil to random coil in aqueous solution, has been examined ultrasonically by several investigators. Despite the fact that many of its physical and chemical properties have been rather well characterized by numerous investigations, and that theoretically this effect should be most pronounced at the relaxation frequency. According to his estimated value of $10^{-7}$ sec as the mean relaxation time at the midpoint of the transition, the mean relaxation frequency is 1.6 MHz. Lewis, on the other hand, was not able to observe the absorption maximum corresponding to the helix coil transition and attributed the observed absorption to solvation phenomena. Saksena, et al., have investigated the absorption maximum but have calculated that the relaxation time is smaller than that predicted by Schwarz by a factor of approximately 10. Zana, et al., have investigated the absorption in nonaqueous solutions of several synthetic polypeptides that were made to undergo helix coil transitions, and, although the results obtained do not indicate that the helix coil transition is the principal mechanism of absorption, definite changes in the absorption are observed with changes in the molecular configuration.

Bovine serum albumin, a globular protein which undergoes a complex conformal change with pH in aqueous solution, was chosen for study because many of its physical and chemical properties have been rather well characterized by numerous investigations. Briefly, the bovine serum albumin molecule has a compact structure within the pH range 4.3 < pH < 10.5 and an expanded structure outside that region. Although the nature of the conformal change has not yet been determined exactly, it is reasonable that the unfolding of the BSA molecule which results in the expanded structure may somehow involve a helix-coil transition. This is suggested by optical rotation and dispersion experiments in which the apparent helix content of bovine serum albumin changes with pH.

**Experimental Section**

Bovine serum albumin (BSA) Fraction V powder, Lot 82268, was obtained from General Biochemicals and maintained at $-7^\circ$ until used. Fraction V grade material was used for this investigation since it is more readily available than the purest grade in the large quantities necessary for this investigation. The solution was prepared by placing the BSA on the surface of a quantity of singly distilled water sufficient to prepare a solution of concentration 0.04 to 0.1 g/cc. The flask was then placed in the refrigerator until mixing of the two components was complete, usually accomplished overnight. The solution was filtered twice through type A glass fiber filters (Gelman Instrument Co.) in order to remove foreign particles larger than 0.3 μ diameter, and after filtration the solution was maintained at 8° until used. Generally, the acoustic experiments were started within a few hours after the solutions were prepared. The concentration of each protein solution was determined with a Beckman Model DU spectrophotometer using the extinction coefficient determined by Cohn, et al., viz., $E_{1\%}^{1cm} = 6.0 \times 10^{-6}$ at 280 μ.

The pH of each solution was changed in steps ranging from 0.2 to 0.5 pH unit by the addition of known quantities of standard volumetric solutions of HCl and KOH. During this procedure, the solutions were stirred gently with a magnetic stirring bar to minimize pH gradients. Measurements of pH were made to within ±0.1 pH unit, in the temperature range 19 to 23°, with a Beckman Model H-2 glass electrode pH meter which was standardized with accurate buffer solutions at pH 4, 7, and 9. Ultrasonic absorption and velocity measurements were not begun on the BSA solutions until at least 15 min after a pH change was made, which allowed sufficient time for the temperature to reach the desired value and also allowed the BSA molecules to reach configurational equilibrium. The titrations were carried out in two stages using separate solutions, i.e., the pH was varied from neutral to about 2.3 for one set of solutions and from neutral to about 11.8 for the second set of solutions. This was considered the maximum pH range allowable to avoid possible damage to the sample chamber. For a particular set of experiments, the neutral solution was examined first, and then, after the investigation at either pH 2.3 or pH 11.8 the solution was titrated back to neutral for comparison with the first measurement.

The amplitude of a plane, progressive sinusoidal wave decays exponentially as it propagates through a lossy, homogeneous, infinitely extended medium according to

$$P(x,t) = P_0 \exp(-ax) \exp(i(\omega t - kx))$$

where $P$ is the instantaneous value of the acoustic pressure amplitude as a function of distance $x$ and time $t$, $\alpha$ is the absorption coefficient, $\omega$ is the angular frequency, and $k$ is the wave number. The pulse technique employed in this study to measure the absorption coefficient in liquids simulates the free field condition

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expressed by eq 1 for finite sample sizes, provided that
the pulse length in the medium is short compared with
the acoustic path length. In addition, the error in the
absorption coefficient due to the spectrum of frequencies
associated with a pulse train is negligible if the pulse is
at least \( 30\pi/\omega \) sec in length.\(^{17}\)

Two techniques were employed to measure the ab-
sorption coefficient to within 5\% over the frequency
range from 0.3 to 163 MHz. The first technique,\(^{18}\) an automated version of that described by Pellam
and Galt,\(^{17}\) can be employed for frequencies greater than 9
MHz, for the transducer diameter available (1 in.),
where diffraction effects are small. Over the frequency
range from 9 to 69 MHz, two matched 3 MHz funda-
mental frequency X-cut quartz transducers are set
parallel and coaxial to each other in the liquid to be
studied. Each transducer is edge mounted with its
front face in direct contact with the liquid and its back
face exposed to an air-filled cavity. One transducer
emits pulses of ultrasonic energy while the other trans-
ducer detects acoustic pulses. The acoustic path length
is varied by displacing one transducer relative to the
other at constant velocity. The amplitude of the re-
ceived acoustic pulse varies according to eq 1 (where
x is the instantaneous acoustic path length) and the
electrical pulse from the receiving transducer is re-
corded on a logarithmic chart recorder whose paper dis-
placement is slaved to the moving transducer. Over
the frequency range 75 to 165 MHz, a pair of 15 MHz
fundamental, X-cut quartz transducers bonded to
fused quartz delay rods are substituted for the 3
MHz transducers. Velocity measurements are per-
formed by electronically measuring the length of time
required to change the acoustic path length by 100
wavelengths of sound. Details of this system can be
found in ref 18 and 19.

At frequencies below 9 MHz, the technique described
above for measuring the absorption coefficient and
velocity of sound requires unreasonably large diameter
transducer elements to minimize diffraction effects.
These effects arise because the requirement of plane
waves, as described by eq 1, is only approximated by a
finite size transducer and the approximation deteriorates
as the wavelength of sound approaches the diameter of
the transducer. A comparison technique, described by
Carstensen,\(^{20}\) in which the acoustic properties of the
sample liquid are determined relative to those of a
known reference liquid, minimizes diffraction effects.
In the present study, water served as a convenient
reference liquid since its absorption coefficient and
velocity of sound have already been determined ac-
accurately.\(^{21,22}\) Two compartments of a double chamber
tank are separated by an acoustically transparent win-

dow. The transducers are mounted coaxially
parallel and are supported a fixed distance apart on a
sliding carriage. The acoustic measurements are made
by varying the relative amounts of sample and refer-
ence liquids in the acoustic path by moving the carriage
along the axis of sound propagation. If the velocity
of sound in the sample liquid is within a few per cent of
that in the reference liquid, then varying the relative
amounts of each within the acoustic path produces
little change in the acoustic path length and conse-
quently only a negligible diffraction effect. Details
of this technique are to be found in ref 19 and 20.

Results
The ultrasonic absorption in aqueous solutions of
BSA was measured as a function of pH from 2.3 to 11.8
at six frequencies ranging from 2.39 MHz to 50.25
MHz at 20\°. The data are presented in terms of the
excess frequency-free absorption per unit concentration,

\[
A = \Delta a/c^2
\]  

where \( c \) is the concentration of solute in grams per cubic
centimeter of solution and \( \Delta a \) is the difference in the
absorption coefficients between the solution and the
solvent. The absorption coefficient of the solvent found
by Pinkerton\(^{21}\) has been used to evaluate \( \Delta a \). In
aqueous solutions of BSA, the excess absorption has
been shown to increase linearly with concentration at
least to about 0.1 g/cc in the pH region where expa-
sion of the molecule is known not to occur,\(^{18}\) Figure 1.

Figure 1. Concentration dependence of acoustic absorption of
BSA at 20.0° in an aqueous solution of 0.15 M KCl.

\(^{19}\) L. W. Kessler, Ph.D. Thesis, Univ. of Illinois, Urbana, Ill.,
1968.
\(^{22}\) M. Greenspan and C. E. Tschiegg, J. Acoust. Soc. Amer., 31, 75
(1959).
This implies that contributions to the absorption arising from possible intermolecular interactions are not important, and that in this region, as far as the ultrasonic wave is concerned the solution may be considered equivalent to one which is infinitely dilute.

The excess frequency-free absorption is plotted as a function of pH for the acid titration in Figures 2, 3, and 4 and it is clear that within experimental error the ultrasonic absorption changes reversibly with changes of pH. This is reasonable on the basis of the previously observed reversibility of other properties of BSA in aqueous solution over the same pH range. The very small effect on the absorption of the formation of KCl as a result of back titration has been corrected in all the figures. As the BSA solution is made acidic, the absorption increases by a small amount until the pH reaches about 4.3. Beyond this point, there is an abrupt increase in A which is markedly greater at lower frequencies than at higher frequencies. The velocity of sound in the solution was also measured as a function of pH at 2.39 MHz and the result for the acid titration is presented in Figure 5. In this figure, $\Delta v$ is the difference between velocity of sound in the solution and that in the solvent, divided by the solute concentration. For the purpose of this calculation, changes in the velocity of sound in water were assumed to be negligible with changes of pH compared with changes in the velocity of sound in the solution. The resulting curve exhibits a minimum at about pH 4.1 which is also the approximate midpoint of the abrupt absorption increase.

Ultrasonic titration on the alkaline side shows a somewhat greater increase in the absorption coefficient than at acid pH, an example of which is shown in Figure 6. As the alkalinity of the solution is increased from pH 7, the variation of A with pH is small up to about pH 10 where A increases rapidly. The alkaline effect is also reversible over the pH range investigated. Further, as with the acid titration, the effect is more pronounced at lower frequencies. The velocity of sound, on the other hand, was found to increase monotonically with increasing alkalinity.

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The frequency dependence of $A$ was measured over the frequency range from 0.3 to 163 MHz by the techniques indicated above and a composite of all the data, at neutral pH, is shown in Figure 7. Over the frequency range covered, a broad distribution of relaxation times may be necessary to characterize the absorption behavior. The presence of velocity dispersion further indicates the relaxational behavior of the absorption over this frequency range, Figure 8. In order to relate changes in the relaxational properties of BSA in aqueous solution with molecular conformational changes, the variation of the absorption coefficient as a function of frequency is examined. It is evident from Figures 2-6 that there are two sharp transition regions, one which is completely delineated in the acid pH region, and a second, in the alkaline pH region, which extends beyond the range of these measurements. If the following definition is made, $A_o = A$(pH 3.5) − $A$(pH 7.0), then the frequency spectrum of $A_o$ is described, approximately, by a single relaxation process as shown in Figure 9. The best fit single relaxation curve was determined by the method of "least squares" for an equation of the form

$$A_o = \frac{a_o}{1 + (\nu/j_0)^{2}} + b_o$$  \hspace{1cm} (3)

where $a_o$, $b_o$, and $j_0$ are constants, $j_0$ being the characteristic relaxation frequency. For eq 3, $j_0 = 2.2$ MHz, $a_o = 543 \times 10^{-16}$ sec$^2$ cc/cm g and $b_o = 2.2 \times 10^{-16}$ sec$^2$ cc/cm g.

The absorption coefficient was determined as a function of temperature from 15 to 38° and at two pH values viz., 7.0 and 2.9. Higher temperatures were avoided since above 40° irreversible denaturation of the molecule can occur.\footnote{23} For absorption due to either shear or bulk viscosity, $\eta$, the temperature dependence of the absorption coefficient is given by Eyring\footnote{24} as

$$a = 2\pi c^2 \eta \exp\left[\frac{\Delta F}{RT}\right]$$  \hspace{1cm} (4)

where \( p_0 \) and \( c_0 \) are the density of the medium and velocity of sound respectively, \( \Delta F \) is the activation energy, \( R \) is the gas constant and \( T \) is the absolute temperature. This relation is valid if the period of the acoustic wave does not approach a characteristic time constant associated with a relaxation process. Table I lists the apparent activation energies determined for a solution of 0.05 g/cc of BSA in water. In all cases, the apparent activation energy is less than that for pure water. At pH 7.0, \( \Delta F \) is independent of frequency, within the experimental error, as it is for water. However, for low pH values, \( \Delta F \) is a strong function of frequency which indicates the presence of additional relaxation phenomena.

**Discussion**

In order to account for the magnitude of the excess absorption coefficient at neutral pH, quantitative consideration is given to (1) dynamic shear viscosity of prolate ellipsoidal molecules in solution; (2) frictional losses associated with relative motion between the solute and solvent particles; (3) scattering of the acoustic waves by the solute particles; and (4) mode conversion of the longitudinal acoustic wave into rapidly decaying transverse acoustic waves at the solute-solvent interfaces. Such consideration shows that, collectively, the above mechanisms of absorption account for only a small percentage of that observed. An investigation of the dynamic shear viscoelasticity of BSA in glycerol and water mixtures was reported by Allis and Ferry in the frequency range from 0.04 to 400 Hz and the dynamic shear viscosity observed did not agree with theories of rotational relaxation. It is suggested by these authors that the origin of the viscoelasticity in BSA does not arise from orientation of the molecule by the shear stresses but primarily from an intramolecular flexibility not otherwise observable. However, a reexamination of the contribution of shear viscosity, taking into account this discrepancy, does not alter significantly the results presented here.

As a result of the above considerations it is concluded that the structural (bulk) viscosity of the solution is the principal factor responsible for the ultrasonic absorption. The distribution of relaxation times and the velocity dispersion indicate structural relaxation and the same mechanism is thought to be present in aqueous solutions of other biomacromolecules, viz., hemoglobin, and dextran. A reasonable description of the observed phenomena has been given by Andrease, et al., for a nonelectrolyte dissolved in water. The short range structure of water rapidly breaks down as solute molecules are dissolved. Water molecules, which bind to the solute molecules in hydration layers, reach an equilibrium state with the unbound solvent molecules and it is this equilibrium which is perturbed by the sound wave and gives rise to absorption.

In order to understand the observed changes of the ultrasonic absorption coefficient that occur with changes in pH, a brief summary of the physical-chemical properties of BSA in aqueous solution is presented. It has been generally recognized that serum albumin molecules undergo marked reversible structural changes as the pH of the environment is altered, although the exact nature of these changes remains unclear. Within the range 4.3 < pH < 10.5, each BSA molecule behaves as an undeformable solid particle whose shape can be approximated by a prolate ellipsoid. Outside this range, it was thought that a simple swelling of the compact globular structure was responsible for the observed increase in optical rotation, in viscosity, and in other physical parameters until Tanford, et al., discovered a distinct stepwise change in the intrinsic viscosity, \( [\eta] \), as the pH decreased below 4.3. Specifically, between pH 4.3 and 4.0, \( [\eta] \) increases sharply by about 22% and between pH 3.5 and 2.8 an 84% increase occurs. This two-step process was observed when the BSA was suspended in 0.15 M KCl and was not observed at low ionic strengths. It should be noted that the ionic strength of the Fraction V material supplied by the manufacturer is high. In nearly the same pH region as the smaller increase in \( [\eta] \), \( A \) increases abruptly between pH 4.4 and 3.8. There is no change in \( A \) corresponding to the 54% increase in \( [\eta] \) below pH 3.5. This suggests that if the same mechanism is responsible for both the first increase in \( A \) and the increase in \( [\eta] \) at pH 4.3, then a separate mechanism which does not affect the ultrasonic properties of the solution is responsible for the larger increase in \( [\eta] \). Tanford, et al., propose that the complete expansion of the molecule by the shear stresses is primarily from an intramolecular flexibility not otherwise observable.

**Table I**

<table>
<thead>
<tr>
<th>pH</th>
<th>f (MHz)</th>
<th>( \Delta F ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>8.87</td>
<td>2.19</td>
</tr>
<tr>
<td>7.0</td>
<td>14.79</td>
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</tr>
<tr>
<td>7.0</td>
<td>26.65</td>
<td>2.26</td>
</tr>
<tr>
<td>2.9</td>
<td>8.87</td>
<td>0.671</td>
</tr>
<tr>
<td>2.9</td>
<td>14.79</td>
<td>1.35</td>
</tr>
<tr>
<td>2.9</td>
<td>26.65</td>
<td>1.70</td>
</tr>
<tr>
<td>Water</td>
<td>All</td>
<td>4.23</td>
</tr>
</tbody>
</table>

sion of BSA occurs in at least three distinct stages. As the pH is decreased a structural change occurs from a compact form to an "expandable form." At lower pH, the molecule expands physically, and for pH < 4, a small time-dependent increase in the viscosity occurs. This last stage is attributed to possible slow aggregation of the molecules.

Aggregation has been observed in aqueous BSA solutions at acid pH by many investigators. According to Williams and Foster, the principal aggregate is the dimer and it has a maximum concentration at pH 3.3. Below pH 3.0 or above pH 3.5, the rate and extent of dimerization is diminished and these investigators conclude that the turbidity, or cloudiness of the solution that occurs below the isoelectric point which is usually attributed to aggregation, is due to the liberation of a lipid impurity that is carried along with the BSA molecule. Since the abrupt change of the ultrasonic absorption coefficient does not occur in the neighborhood of pH 3.5, it is unlikely that the acoustic disturbance is associated with dimerization. If the impurity itself was involved in the absorption mechanism, then some correlation should have been observed with liberation of this material, which occurred above pH 4.3.

The stepwise expansion process and more recent evidence of a two-step change in the rotational relaxation time occurring at pH 4.1 and pH 3.6 support the N-F transformation theory proposed by Aoki and Foster. The N state is the compact rigid form of the molecule which exists between pH 4.3 and 10.5. It is thought to be composed of two pairs of globular subunits held together tightly with each member of a pair held to its partner by hydrophobic bonds. The two pairs of subunits are then bonded to one another electrostatically to make up a four unit globule. This is the so-called F state or "intermediate F" form. This state, which corresponds to the absorption increase at acid pH over that at neutral pH, is due to the liberation of a lipid impurity that is carried along with the BSA molecule. Since the abrupt change of the ultrasonic absorption coefficient does not occur in the neighborhood of pH 3.5, it is unlikely that the acoustic disturbance is associated with dimerization. If the impurity itself was involved in the absorption mechanism, then some correlation should have been observed with liberation of this material, which occurred above pH 4.3.

Aoki and Foster, Williams and Foster, and others have shown that the elastic moduli and densities of these units remain constant.

The transformation F' → F is too slow to be observed with the ultrasonic frequencies employed in this investigation.

Figure 5 shows that the reduced velocity exhibits a minimum at pH 4.1. If the expansion of BSA occurs by separation of the globular units without certain changes to the globular units themselves, as implied by the fragmentation experiments of Weber and Young, then it may be assumed, to a first approximation, that the elastic moduli and densities of these units remain constant. Urick, under the simplifying assumption that a solution can be considered homogeneous to the sound wave, derived the following equation for the velocity of sound in a solution, $C_s$.

$$C_s = C_0 \left( \frac{1}{(1 + \phi \sigma_K)(1 + \phi \sigma_\rho)} \right)^{1/3}$$

where

$$\sigma_K = \frac{K_0 - K_s}{K_0}; \quad \sigma_\rho = \frac{\rho_1 - \rho_0}{\rho_0}$$

In eq 5, the subscripts 0 and 1 correspond to the solvent and solute, respectively, $K$ is the bulk modulus, $\rho$ is the density, and $\phi$ is the volume fraction of the solution occupied by solute. At 2.39 MHz, pH 7.0 and 20°, $C_0 = 1482.4$ m/sec and $C_s = 1506.5$ m/sec when the solute concentration is 9.18%. If a reasonable value for $\rho_1$ is taken as 1.23 (see page 307 of ref 1) then $\sigma_\rho = 0.33$ and $\sigma_K = -0.77$. If $K_0$ is 2.18 $\times$ 10^5 N/m^2 then the bulk modulus of BSA is 3.86 $\times$ 10^5 N/m^2. It follows from eq 5, under the assumption stated above, that the minimum at pH 4.1 corresponds to a net decrease in the volume fraction of molecules in solution.

References:

which could result from a reduction of the protein hydration. This is reasoned physically as follows. In the N state, each gram of protein is associated with approximately 0.2 g of bound water molecules. If, when the molecule assumes the intermediate F' state, the hydration layer remains intact, then the internal rotational freedom possessed by the separated globular units would be suppressed and a decrease in the rotational relaxation time would not be observed. As pH decreases below 4.1, the velocity of sound increases monotonically due, in part, to the increased velocity in the solvent alone, and, in part, to the changes in the chemical nature of the solvent and solute at low pH.

The change in the ultrasonic absorption coefficient for pH > 10.5 is more difficult to correlate with molecular events than it is for pH < 4.3 since very little experimental work on the nature of the alkaline expansion has been reported in the literature. Weber and Tanford, et al., suggest that both the acid and alkaline expansions are similar; however, no other experimental evidence has been presented to substantiate this. Abrupt decreases of the rotational relaxation time of BSA were observed by Weber at pH 3.6 and pH 11.2. It has been observed that at pH 3.6, BSA begins to expand rapidly with decreasing pH, and thus it is reasonable to assume that expansion is also rapid with pH changes above 11.2. The ultrasonic titrations at acid pH and alkaline pH are similar in that the absorption coefficient begins to increase above pH 3.6 and below pH 11.2, i.e., before the molecule actually expands. For pH < 3.6, no correspondence is evident between expansion and A; however, for pH > 11.2, A is increasing sharply which implies that the mechanisms responsible for the absorption increase may be different in each region. Further evidence to support this is the sharp contrast between the velocity titrations at acid and alkaline pH. Tanford and Buzzell have measured [9] to pH 10.5 and found that no observable change occurs between pH 9.3 and pH 10.5 whereas here it has been shown that A increases significantly. Further correlation of the ultrasonic absorption increase for pH > 10.5 must await further details of the nature of the alkaline expansion.

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