Influences of structural factors of biological media on the acoustic nonlinearity parameter B/A

Jian Zhang
Bioacoustics Research Laboratory, Department of Electrical and Computer Engineering, University of Illinois at Urbana–Champaign, 1406 West Green Street, Urbana, Illinois 61801

Mark S. Kuhlenschmidt
Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Illinois at Urbana–Champaign, 2001 South Lincoln Avenue, Urbana, Illinois 61801

Floyd Dunn
Bioacoustics Research Laboratory, Department of Electrical and Computer Engineering, University of Illinois at Urbana–Champaign, 1406 West Green Street, Urbana, Illinois 61801

(Received 29 May 1990; accepted for publication 17 September 1990)

The influence of structural factors of biological media on the acoustic nonlinearity parameter B/A have been studied at the tissue, cellular, and molecular levels, using the thermodynamic and finite amplitude methods. B/A was determined as the structural factors of the media were altered physically and biochemically, while chemical composition was maintained unchanged. Significant structural dependencies of B/A were observed at all three levels; 26% of the dry weight contribution to the total B/A (the B/A value with water contribution subtracted) is due to the cell–cell adhesive force in liver tissue, 20% is due to the hepatocyte cellular structure, and 15% is due to secondary and tertiary protein structure.

PACS numbers: 43.25.Ba, 43.80.Cs

INTRODUCTION

The acoustic nonlinearity parameter B/A has been studied in various biological media (Everbach, 1989; Law *et al.*, 1981, 1985; Gong *et al.*, 1984; Sehgal *et al.*, 1984; Cobb, 1982; Dunn *et al.*, 1981), and its relationship with chemical composition of media has been investigated (Everbach, 1989; Apfel, 1983, 1986; Sehgal *et al.*, 1986a). It has been found that the B/A in biological media ranges from 5–12, and is a linear combination of the contributions due to various chemical components. It has been shown that no significant difference exists between the *in vivo* and *in vitro* state (Zhang and Dunn, 1987). However, no systematic study has been reported regarding the influence of structural features of the media on the B/A parameter, though there is evidence to suggest such dependence (Law *et al.*, 1985). This paper reports such a study. Both the thermodynamic and the finite amplitude methods were employed to measure the relative B/A changes when three structural levels, i.e., tissue, cellular, and molecular levels, were perturbed physically and biochemically. The study was arranged so that only the structural features of the specimen were altered, with the chemical composition maintained unchanged. It has been found that about 26% of the dry weight B/A (the B/A value obtained with the water contribution subtracted away) is due to tissue level structure, 20% due to the cellular level structure and 15% due to the molecular structure of protein (Zhang, 1990).

I. B/A MEASUREMENT METHODS

A. Thermodynamic method

A 4-ml thermodynamic measurement system was developed to measure the B/A values of small sample preparations such as hepatocyte suspensions of aqueous solutions of protein (Zhang and Dunn, 1991; Zhang, 1990). Briefly, this system employed a velocimeter to monitor the change of speed of sound in a sample during a process in which the ambient pressure of the velocimeter was decreased rapidly. The relative change of the speed of sound with respect to pressure can be used to calculate the B/A, as given by the relationship (Beyer, 1974),

$$\frac{B}{A} = 2\rho_0 c_0 \left(\frac{\partial c}{\partial p}\right)_s, \tag{1}$$

where ρ_0 is the ambient density of the medium, c_0 is the infinitesimal speed of sound, and $(\partial c/\partial p)_s$ is the derivative of the speed of sound with respect to pressure for an isentropic process.

B. Finite amplitude method

The finite amplitude measurement system was modified from that previously employed (Law, 1984; Law *et al.*, 1985). The method determines B/A by measuring the second harmonic acoustic pressure as

 0001-4966/91/010080-12$00.80

$$p_2(x) = \frac{\pi f(2 + B/A)}{2\rho_0 c_0^3} p_1^2(0) x e^{-(\alpha_1 + \alpha_2/2)x} \mathrm{DIFF}(x), \quad (2)$$

where x is the distance between the receiving and transmitting transducers, $p_1(0)$ is the acoustic pressure output of the transmitter at the fundamental frequency f, $p_2(x)$ is the amplitude of the second harmonic acoustic pressure averaged over the surface of the receive transducer, DIFF(x) is the diffraction correction that is a function of the geometry, ρ_0 is the equilibrium density of the medium, and c_0 is the infinitesimal wave speed. Here α_1 and α_2 are the absorption coefficients of the medium at the fundamental and second harmonic frequencies, respectively.

Figure 1 shows the block diagram of the measurement system. The Wavetek 271 pulse generator produces a sinusoidal burst of 15 cycles at 3 MHz which, after being amplified (Amplifier Research, Model 10LA), drives the transmitter. The two channels of the Tektronix 2430A digital storage oscilloscope acquires the transmitted and received pulses, which are then transferred to the AT&T 6300 computer, via an IEEE 488 interface. Spectrum analysis and time delay calculations are then made, respectively, for the fundamental frequency components at 3MHz and its 6-MHz second harmonic (Zhang, 1990), and for the speed of sound.

In order to improve the accuracy of the B/A measurement, and to circumvent the very involved transducer calibration procedures (Law *et al.*, 1985), a comparative procedure was employed to obtain the relative B/A measurement. The method compares the second harmonic pressure component determined for a sample with that of a reference medium with a known B/A value as follows.

Assuming that p_0, p_1, and p_2 are the transmitter output pressure, the fundamental acoustic pressure and the second harmonic pressure at distance x from the transmitter, respectively, and that v_0, v_1, and v_2 are, respectively, the fundamental driving voltages of the transmitter, and the fundamental and second harmonic voltage components in the received pulse, then the sensitvities of the transducers can be expressed as

$\eta_0 = p_0/v_0$, for the transmitter sensitivity at 3 MHz;

$\eta_1 = p_1/v_1$, for the receiver sensitivity at 3 MHz;

$\eta_2 = p_2/v_2$, for the receiver sensitivity at 6 MHz.

Further if the reference medium is chosen such that its acoustic impedance is close to that of the sample, then it can be shown that (see the Appendix)

$$\frac{(B/A + 2)/(\rho_0 c_0^3)}{(B/A + 2)^*/(\rho_0 c_0^3)^*} = \frac{v_2}{v_2^*} \left(\frac{v_1^*}{v_1}\right)^2 \frac{x^* \mathrm{DIFF}(x^*)}{x \mathrm{DIFF}(x)}, \quad (3)$$

where the * denotes the value for the reference medium. Additionally, let

$$K \equiv \rho_0 c_0^3 v_2 / v_1^2 x \mathrm{DIFF}(x), \quad (4)$$

then,

$$\frac{(B/A + 2)}{(B/A + 2)^*} = \frac{K}{K^*}. \quad (5)$$

From determination of the quantity K for the samples studied and for reference media of known B/A values, the B/A value for the unknown sample can be determined.

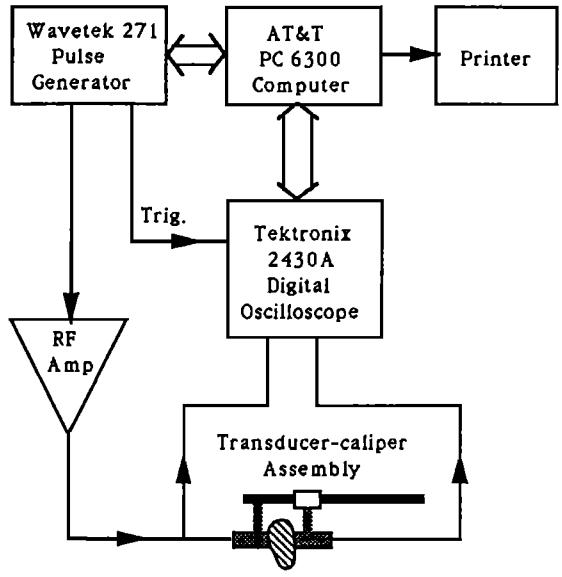

FIG. 1. The block diagram for measurement system of the finite amplitude method.

The transducer sensitivities η_0, η_1, and η_2 change slightly with the acoustic impedance of the media; about 5% for an impedance change from 1.6×10^5 to 1.7×10^5 g/cm^{2}s (Law, 1984). For a 10% NaCl reference solution having an impedance of 1.66×10^5 g/cm^{2}s, and with the cat liver with impedance of 1.64×10^5 g/cm^{2}s, the error introduced by making the above assumption is about 2%.

The measurement system and procedure was tested using a 10% aqueous NaCl solution as a reference whose B/A value was determined to be 6.08 by the thermodynamic method, and using a 23% aqueous BSA solution (impedance 1.67×10^5 g/cm^{2}s) as the unknown. The B/A of the BSA solution was determined to be 6.12, as compared with 6.31 determined by the thermodynamic method.

II. PREPARATION AND EXPERIMENTAL PROCEDURES

A. Specimen preparation

Random source cats of 6–9 lb were anesthetized by i.p. injection with sodium pentobarbital (Nembutal, Abbott Laboratories) at the dose of 0.3 ml/lb. Male Sprague–Dawley SD rats were obtained from Harlan Sprague Dawley Inc., Indianapolis, Indiana, were used when about 500 g, and were anesthetized by Metofane inhalation (Pitman–Moore, Inc., NJ). Anesthesia was confirmed by lack of pedal response to mechanical stimulation. The abdomen was then incised, and the liver isolated for B/A measurements. Cats were sacrificed by i.v. injection of T-61 euthanasia solution (Taylor Pharmacal Co., Decatur, IL, distributed by American Hoechst Corp. Somerville, NJ) at 0.14-ml/lb body weight. The liver specimens obtained from the rats weighed about 15 g. The left lobes weighed about 6 g, the volume was

about 6 ml, and the thickness ranged from 0.6–0.9 cm, sufficient for adequate transducer separation and measurement.

B. Tissue perfusion procedure

In vitro perfusion of the liver, first in the absence of calcium and then in the presence of collagenase, was first used by biochemists to obtain large yields of hepatocytes (Seglen, 1973, 1976). This procedure was adapted in this study to provide a gentle method for the gradual dissociation of the intercellular adhesive interactions among hepatocytes. The procedure is described briefly as follows.

It is well documented, in a variety of species (Obrink *et al.*, 1977; Seglen, 1973, 1976; Kawaguchi *et al.*, 1981; Kuhlenschmidt *et al.*, 1982), that upon loss of tissue calcium hepatocytes lose intercellular adhesive contacts, begin to "round-up" or change from a cuboidal to a more spherical morphology, and remain attached to each other only by the tight junctions and gap junctions (Rochmans *et al.*, 1975). Following subsequent perfusion with collagenase, the cell contacts are digested, the cells become separated from each other, and single hepatocyte suspensions can be isolated with only gentle mechanical agitation of the liver following removal Glisson's capsule (Seglen, 1973, 1976). Morphologically, at the electron microscope level, the unperfused liver tissue shows intact intercellular adhesion; the membranes from two neighboring hepatocytes remain an intimate contact and parallel to each other. The liver tissue perfused with Ca^{++}-free buffer is seen to contain spherically shaped cells and partially dissociated cell–cell contacts which include gap and tight junctions. At the end of the perfusion procedure with collagenase buffer, only cell cords remain. Complete dissociation of these cell cords can be achieved by gentle mechanical treatments, such as massage or rolling the cell suspension in a round-bottom flask.

The perfusion arrangement is shown in Fig. 2. The pump (Masterflex, catalog number 7553-10, Cole–Parmer) delivers perfusate, at the rate of 76 ml/min for cats and 56 ml/min for rats, into the liver via a bubble trap, which prevents air being pumped into the specimen. The perfusate returns to the reservoir and is recirculated. A graduated cylinder serves as a convenient reservoir for monitoring the amount of perfusate intake into the liver.

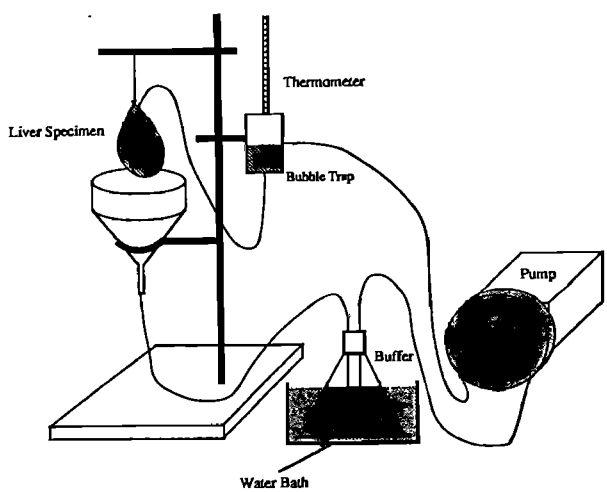

FIG. 2. Schematic diagram of liver perfusion apparatus.

Three kinds of perfusates were used, viz., the physiological buffer, the Ca^{++}-free buffer, and the collagenase buffer. The physiological buffer was prepared using powdered cell culture medium (Eagle Medium-GIBCO Laboratories) containing 2 mM $CaCl_2$, *p*H 7.5. The Ca^{++}-free and the collagenase buffers were prepared from the following recipes:

Ca^{++}-free buffer:

8.3 g/l NaCl, 0.5 g/l KCl,

2.4 g/l HEPES, 4 mM EDTA, *p*H 7.4,

Collagenase buffer:

3.9 g/l NaCl, 0.5 g/l KCl,

0.7 g/l $CaCl_2 \cdot H_2O$, 24.0 g/l, HEPES,

15.0 g/l, BSA, Cohn Fraction V, Sigma, *p*H 7.6.

The Ca^{++}-free buffer was stored at refrigeration temperature (about 4 °C to 5 °C) and the collagenase buffer was stored frozen until used. Collagenase (type IV, Sigma) was added to the collagenase buffer, at the concentration of 0.8 mg/ml, at the time of use to prevent loss of activity during storage.

The perfusion procedure began with physiological buffer, which was allowed to pass through the liver directly to waste, thereby flushing all blood from the organ before switching over to recirculation. The perfusion of the physiological buffer was not expected to alter the structure of the liver tissue and thus provided a control state. After a period of time, usually between 20–60 min, perfusion with Ca^{++}-free buffer was initiated. Caution was exercised not to introduce air bubbles into the liver during the switch over from one buffer to the other. The perfusion of the Ca^{++}-free buffer lasted about 60–90 min, before the perfusion with collagenase buffer was initiated.

C. Hepatocyte suspension preparation

The hepatocyte suspension was obtained by gentle messaging of the liver, perfused as described above, in a small plastic bag. The suspension was first concentrated by sedimentation at unit gravity for 10–15 min, to allow preferential settling of hepatocytes. The supernatant was removed by pipetting, and the concentrated hepatocyte suspension was then degassed for 1½ h. The suspension was transferred carefully to the velocimeter using a transfer pipette, so that air bubbles were not introduced. The *B/A* determination followed, after which the hepatocyte suspension was transferred back to a test tube and added to the unused portion of the sample to compensate for the loss in sample handling. The suspension was then subjected to the sonication proce-

dure, and further degassing, in preparation for the B/A determination.

Weight to weight percentage concentration was determined by placing a preweighed sample in an oven at 120 °C for 20 h, after which the dehydrated sample was weighed, and the difference in weight taken to be the amount of water evaporated.

The procedure used to concentrate the hepatocyte suspension was essential because settling moved some hepatocytes out of the acoustic field (the sample chamber was oriented horizontally and the diameters of the transducers are smaller than that of the chamber). The B/A value so determined would have been less than it should have been without settling.

D. Sample sonication

A high intensity sonicator (model 375, Heat System, NJ) was used to destroy hepatocyte cellular structures in order to study the contribution of their structural level. The sonicator was operated at 50% total output power and 80% duty cycle for 1 min. Examinations of the hepatocyte samples under a light microscope (Nikon AFX–II, magnification 100), before and after sonication, confirmed destruction of the cellular structure. The presonicated samples had intact hepacytes freely floating in suspension, occasionally with a few other cells attached, whereas the postsonicated samples exhibited no intact cells and only small fragments of cell membranes.

E. Sample degassing

Small air bubbles within a sample are known to increase the B/A value significantly (Everbach, 1989). Therefore, before each measurement proper, the liquid sample was degassed in a rotary evaporator capable of creating and sustaining a vacuum of 760 mm Hg. Depending upon the surface tension of the liquid, the degassing time varied from 30 min to 2 h. Usually a large quantity of bubbles emerged from within the liquid at the beginning of the degassing process, and as the process continued, the number of bubbles emerging decreased. Highly viscous samples, such as sonicated hepatocyte suspensions in which the sonication process produces a large quantity of bubbles, required several cycles of on–off vacuum first to remove the large air bubbles. An empirically derived criteria for determining the completeness of the degassing procedure was 1/2 h after no further visible bubbles appeared in the liquid.

F. Denatured protein sample preparation

Bovine serum albumin (BSA) solutions were employed to investigate the influence of the higher-order protein structure on B/A. The alterations of the tertiary and secondary structure were produced by the denaturing agent sodium dodecyl sulfate (SDS) (Weber and Osborn, 1969). SDS solutions, of concentration ranging from 0%–3% were prepared using 0.1-M phosphate buffer solution, and adjusted to pH 7.0. BSA (Sigma, St. Louis, MO) solution of 0.1 g/100 ml was then added. SDS solutions, without the addition of BSA, served as controls. All the samples were degassed for 1.5 h before being loaded into the velocimeter for B/A measurement.

Ribonuclease A (Sigma, St. Louis, MO) samples were also prepared for heat denaturation experiments. The protein was dissolved in water, buffered by 1-M glycine and adjusted to pH 2.77 by addition of HCl. Denaturation under these conditions was confirmed by measurement of the optical extinction coefficient at 287 nm by spectrophotometry (Beckman model DU-50). The result is shown in Fig. 3 for protein concentrations of 0.04%, 0.1%, and 0.2% and shows that the protein denatures at about 42 °C and that the denaturation, as observed by the optical extinction coefficient, is independent of the protein concentration. The samples were degassed for 1.5 h before the measurement.

G. Protease digestion sample preparation

BSA solutions in the presence of protease were used for the investigation of the influence of protein primary structure on the B/A. The protease solutions were made by dissolving 4.8 mg of protease (Pronase, Sigma Chemical Company) in 0.5 ml of water, and the BSA solution by dissolving 100 mg of BSA in 20 ml of phosphate buffer solution (8.00 g/L NaCl, 0.20 g/L KCl, 0.20 g/L KH_2PO_4, 0.15 g/L Na_2HPO_4 and pH 7.35). The BSA solution was adjusted to pH 7.62 and degassed. The protease solution was not degassed due to its small volume. The digested protein solution was tested following 8 h digestion by 7.5% SDS-gel electrophoresis for its completeness, as shown in Fig. 4. At the time of B/A measurement, 0.5 ml of protease solution was first introduced into the 4-ml velocimeter, which was then filled with the BSA solution. The sample chamber was sealed and the sample mixed by revolving the velocimeter by hand. The final concentration of BSA was determined to be 43.1 mg/ml, and that for proteases was 1.33 mg/ml. The mixture under these conditions was known to undergo protein digestion (Sprio, 1965). A 50-mg/ml BSA solution and a glycine solution of 50 mg/ml were prepared in phosphate buffer solution by the same procedure as above, but without protease, to serve as controls.

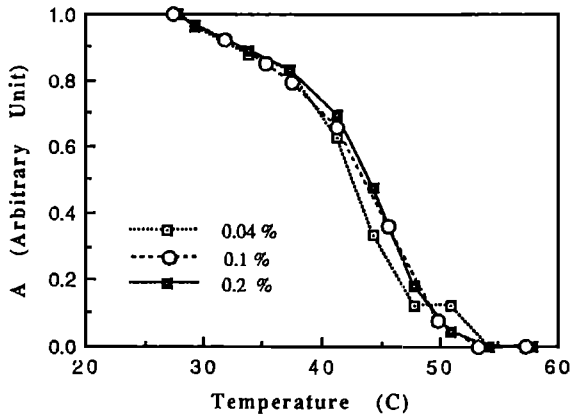

FIG. 3. Normalized extinction coefficient at the optical wavelength of 287 nm during the heat denaturation of ribonuclease A.

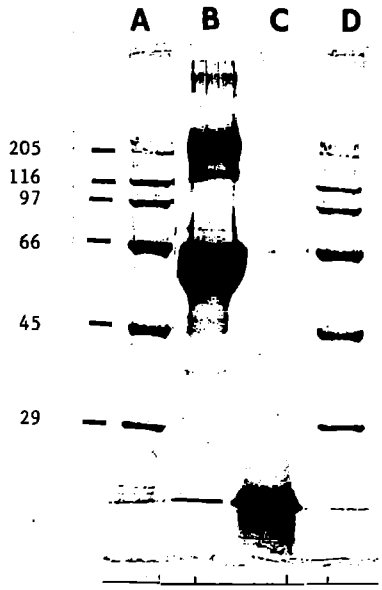

FIG. 4. SDS gel electrophoresis of BSA before and after Protease digestion. BSA was incubated in the presence and absence of Pronase and aliquots analyzed for the degree of digestion by SDS gel electrophoresis as described in Sec. II. Lane A, molecular weight markers; lane B, BSA incubated in the absence of Pronase; lane C, BSA incubated in the presence of Pronase; lane D, molecular weight markers (numbers indicate molecular weight in thousands).

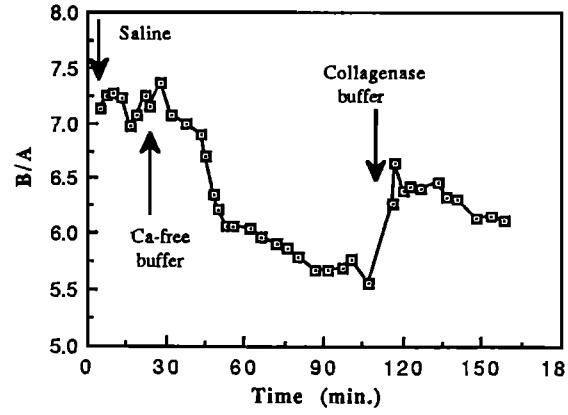

FIG. 5. The B/A values of a cat liver specimen as a function of time at the diferent stages of perfusion.

III. RESULTS

A. Effect of intercellular adhesion

Cat and rat liver specimens were prepared as described above. The liver specimen was isolated, following the surgical procedure, and the portal vein canulated. A site for observation was chosen relatively free of large blood vessels, but sufficiently thick that standing waves could not be established, and the transducers were attached for the measurement procedure. The distance between transducers was adjusted so that they pressed against the liver surface sufficiently tight to prevent an air layer between transducers and the specimen surface, but loose enough not to obstruct the perfusate flow through the sinusoid spaces. The chosen observation site remained unchanged throughout the entire procedure, so that spatial variation of the B/A parameter would not influence the results.

The finite amplitude method was employed. Figure 5 shows a typical B/A data set obtained during an *in vitro* perfusion of a cat liver specimen. It is seen that the B/A value does not vary significantly during perfusion with physiological saline for 20 min. However, the B/A value decreases significantly as Ca^{++} ions are removed from the liver, i.e., during 85 min of perfusion with Ca^{++}-free buffer. When perfusion with collagenase buffer begins, 115 min after initiation of the perfusion procedure, the B/A value increases sharply and then decreases. The trend for B/A to decrease during the perfusions with the Ca^{++}-free and collagenase buffers are considered to be related to the breakdown of intercellular adhesive contacts. The question of whether the decreases in B/A value could be due to alternations in perfusate flow, turbulence, or other mechanical factors was tested by considering different perfusion schedules. Figure 6 shows the perfusion with physiological saline extended to 2 h with no significant B/A change, though the B/A value begins decreasing after initiation of the perfusion with Ca^{++}-free buffer. This demonstrates that the decrease in the B/A is not due to mechanical damage in the liver, but is a biologically relevant event, due to the dissociation of cell–cell contacts. (The increase in the B/A value on initiation of the collagenase buffer is due to the buffer protein content, discussed below.)

Large male Sprague–Dawley rats were used to provide more homogeneous preparations due to control over pathological states.

Figures 7 and 8 show typical B/A parameter and speed of sound profiles obtained for a rat liver during the *in vitro* perfusion procedure. Although B/A is seen to decrease, it also exhibits a significant amount of noise. The sliding average smoothing technique, with a smooth width of 5, applied to the data provides the smoothed version shown in Fig. 9 (Rafferty and Norling, 1986). The velocity data was relatively free of noise.

Figure 10 shows the B/A and speed of sound result for six rat liver specimens. Table I is a tabulation of the results,

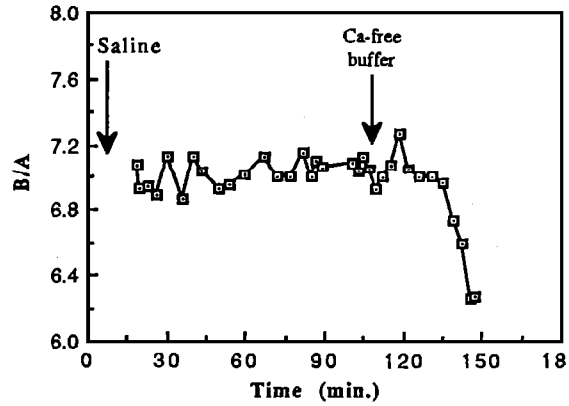

FIG. 6. The plot of B/A versus time for perfusion with saline.

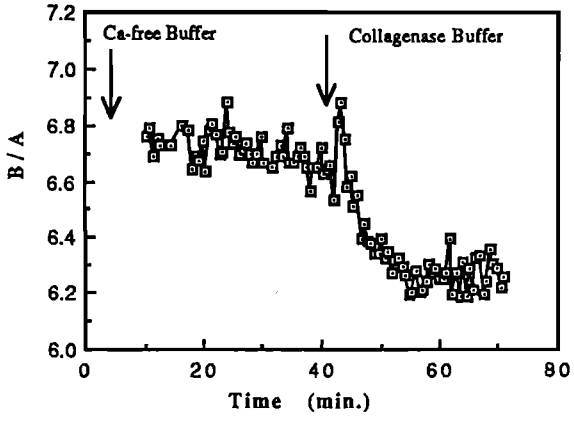

FIG. 7. The B/A data obtained in a rat liver specimen during the perfusion procedure.

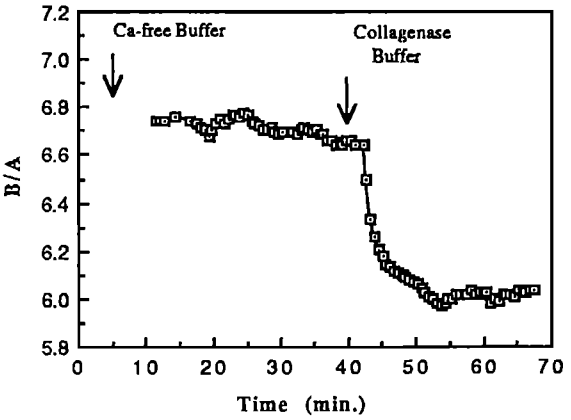

FIG. 9. Smoothed version of Fig. 7 making the B/A decrease more apparent.

showing that while rates and magnitudes of the B/A decrease due to the introduction of the two buffers varies from animal to animal, they exhibit consistency as the intercellular adhesive force is destroyed. An average of 0.68 is obtained from the $\Delta(B/A)$ column, which accounts for 38% $[0.38 = 0.68/(7.0 - 5.2)]$ of the total B/A, on taking the B/A for liver tissue as 7.0 and that for water B/A as 5.2.

The velocity of the rat liver decreases during perfusions with the Ca^{++}-free buffer and collagenase buffer. However, introduction of collagenase buffer causes a sharp increase in the velocity (also in the B/A value). The increases are considered to be due to the proteinaceous content of the collagenase buffer, viz., 2.4% (w/w) HEPES and 1.5% (w/w) BSA which is known to increase velocity (and B/A) (Law *et al.* 1985). The magnitude of the velocity discontinuity Δc_d caused by the addition of BSA and HEPES can be estimated to be

$$\Delta c_d = 0.7 \times (0.024 + 0.015) \times h \simeq 8.1 \text{ (m/s)},$$

where h is the slope of the velocity increase with the protein concentration determined to be 296 m/s (Law, 1984), and 0.7 represents the water content of liver, which was replaced by the collagenase buffer upon its introduction. This estimate is comparable in magnitude to the entries of Table I, supporting the view that the sharp increase in velocity upon introduction of the collagenase buffer is due to its HEPES and BSA contents.

The cumulative velocity decreases during perfusion with both buffers, without taking into account the discontinuity, are also tabulated in Table I as Δc column.

Without mechanical constraint, liver volume was observed to increase by 1.5 to 2.0 ml from the initial volume of about 6 ml, due to the morphological changes. The B/A changes measured under these circumstances reflect the structural change, as well as the decrease in dry weight concentration. The transducers, in the above described procedures, partially prevented a portion of specimen volume from enlargement. This volume increase must be known before the net B/A decrease due to structural changes can be estimated.

Thus, assume the liver enlargement to be homogeneous and isotropic. Then the relative volume increase $\Delta V/V$ will be proportional to the solid angle of the unconstrained portion of the liver tissue; i.e.,

$$\frac{(\Delta v/v)}{(\Delta V/V)} = \frac{\phi}{\Phi}, \tag{6}$$

where $(\Delta V/V)$ and Φ are the relative volume increase for a completely unconstrainted liver, which is 2 ml/6 ml, or 33% and the solid angle defined by the unconstrained portion 4π steradians, respectively; $(\Delta v/v)$ and ϕ are the relative volume increase for a partially constrained liver and the solid angle defined by the unconstrained portion, respectively. Considering the cylindrical volume between the two transducers with diameter of 1.5 cm and the transducer separa-

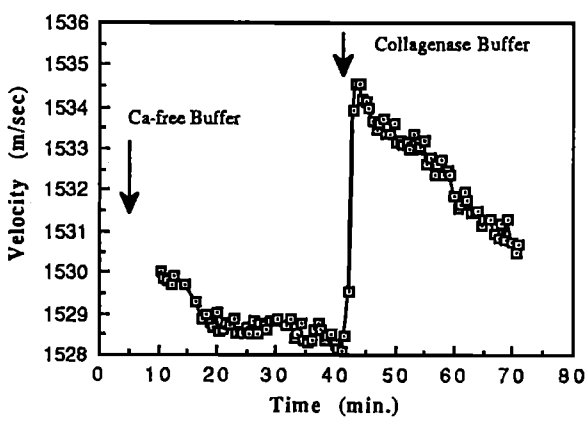

FIG. 8. Speed of the sound measured at the same time as the B/A values of Fig. 7 were obtained, showing decrease of speed of sound during the perfusion process.

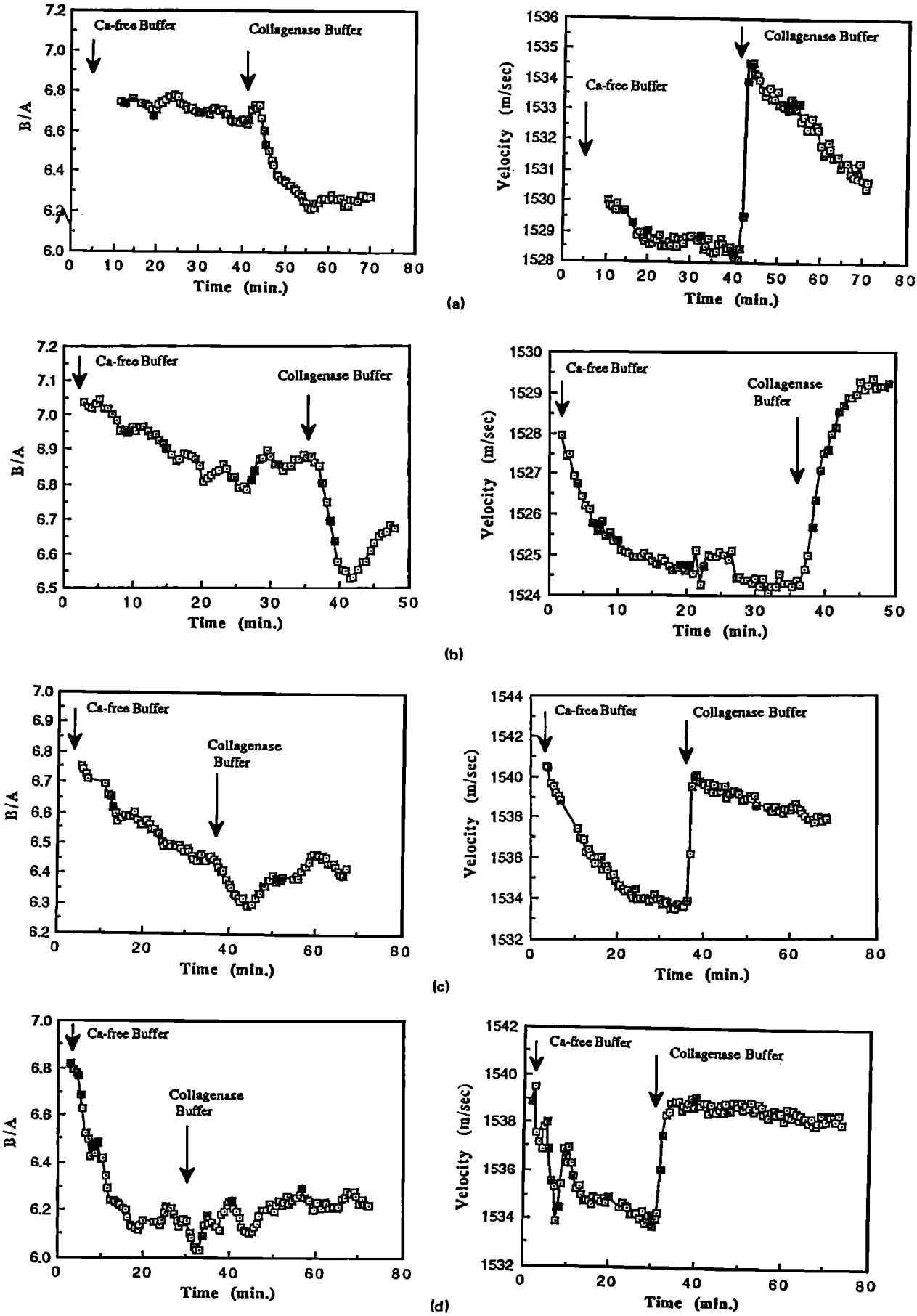

FIG. 10. (a)–(f) B/A and velocity data collected from six rat liver specimens, with the B/A profiles on the left column and the velocity profiles on the right (column). The arrows indicate the onset of the perfusions.

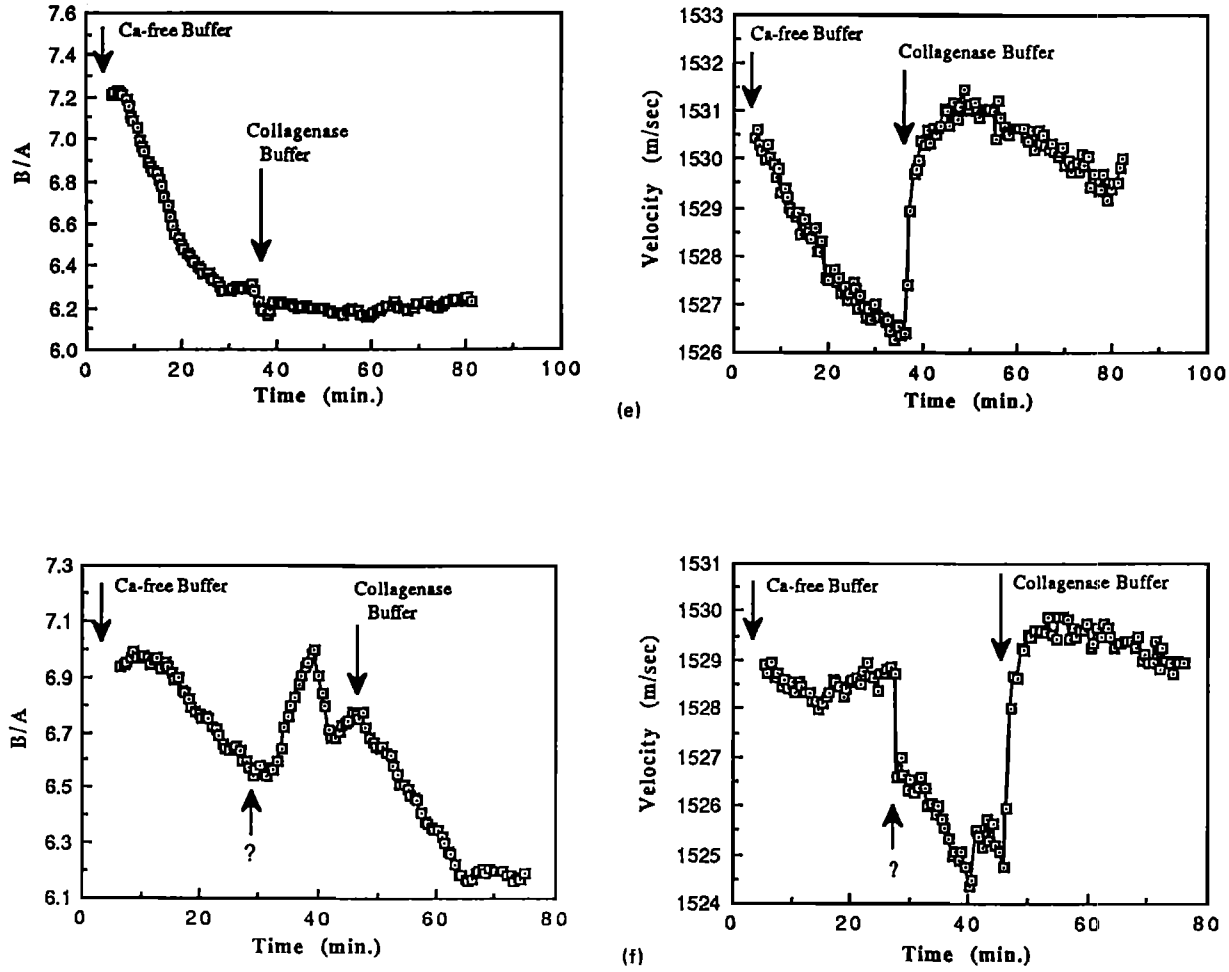

FIG. 10. (*Continued.*)

tion of 0.6 cm, as shown in Fig. 11, then v and ϕ can be calculated to be 1.06 cm^3 and 1.49π steradians, respectively, and according to Eq. (6), $(\Delta v/v) = 0.12$. Thus concentration and B/A decrease 12% because of the volume increase in the portion of the liver between the transducers. Subtracting this B/A decrease due to volume increase from the total B/A decrease of 38% estimated above without consideration for the specimen enlargement due to perfusion, the net decrease is 26%.

TABLE I. Magnitude of B/A and velocity change.

Rat No.	$-\Delta(B/A)$	Δc_d (m/s)	$-\Delta c$ (m/s)
1	0.5	6	5.5
2	0.4	5	3.5
3	0.5	6	9.0
4	0.7	5	5.0
5	1.1	5	7.0
6	0.9	5	5.0

B. Effect of hepatocyte cellular structure

This experiment was designed to assess the change in the B/A value due to hepatocyte cellular structure. Hepatocyte suspensions were prepared as described above and the B/A value determined using the thermodynamic method

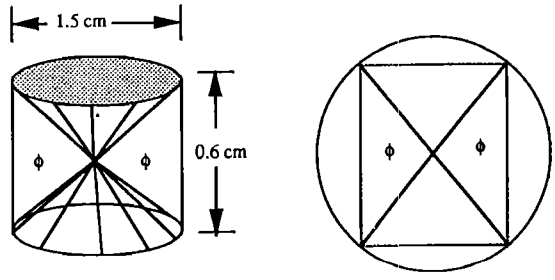

FIG. 11. Illustration of the portion of liver between the transducers and solid angle ϕ.

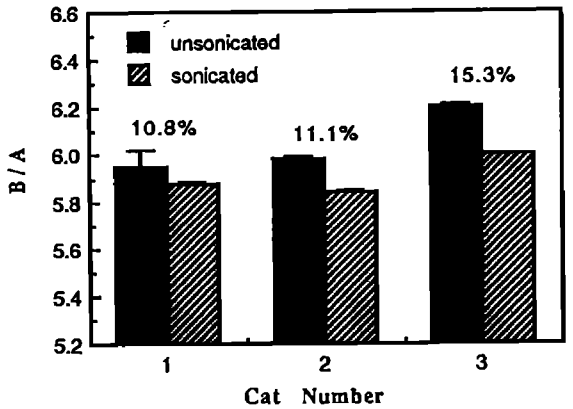

FIG. 12. The B/A values of cat hepatocyte suspensions with and without sonication treatment.

(Zhang, 1990; Zhang and Dunn, 1990).

Figure 12 shows the results of B/A measurements in hepatocyte suspensions of different concentrations, before and after the sonication treatment. The B/A values of the unsonicated samples are consistently greater from 0.08 to 0.15 B/A units. The major difference between the sonicated and unsonicated sample is the missing cellular structure from the sonicated hepatocytes and demonstrates that the B/A parameter decreases as the hepatocyte cell structure is destroyed.

If the results in Fig. 12 are extrapolated to the dry weight concentration of 30%, i.e., to that which exists in the intact liver, the average B/A decrease would be 0.36 units, which accounts for 20% of contribution to the total B/A of the liver tissues, 7.0 [$0.20 = 0.36/(7.0 - 5.2)$].

C. Effects of protein tertiary and secondary structure

The above findings on the dependence of B/A on tissue and cellular level structure also suggests that molecular level structure may also be influential. BSA was employed, with alteration of the tertiary and secondary structure produced by the denaturing agent sodium dodecyl sulfate (SDS) (Weber and Osborn, 1969).

BSA solutions were prepared with SDS ranging from 0%–3%, and solutions of SDS only were controls. The 4-ml thermodynamic method was used for B/A measurement. Figure 13 shows the B/A values of the samples with and without BSA at the different SDS concentrations. The difference in B/A values between the two curves is considered to be due to the contribution of the intact BSA molecule. The sample containing BSA has a greater B/A value because protein increases the B/A value of the solution. The B/A value of 5.7 in the BSA buffered saline alone, i.e, at the 0% SDS concentration, is in agreement with that obtained by other investigators (Law *et al.*, 1985; Cobb, 1982). Figure 14 shows the difference in B/A values following subtraction of the SDS buffer control for the different SDS concentrations. The difference decreases as the SDS concentration increases, and the change levels off at the SDS concentration of about 1%. This decrease of 0.08 B/A units represents 15% of the excess B/A. If it is assumed that this B/A value difference is due to the presence of BSA molecules in the buffer, then the changing state of the BSA molecules at the higher SDS concentrations is being ovserved in the B/A parameter. Assuming the B/A value associated with the intact BSA protein is greater than that associated with the denatured molecule, then the experimental result can be explained as follows. With no SDS molecules present in the solution, the higher-order structure of all the BSA molecules is intact and the B/A value of the buffer solution is high. As more SDS is added, more BSA molecules are denatured and the B/A value decreases. The B/A value finally decreases to the minimum value when sufficient SDS is present in the solution to denature all the BSA molecules. The experiment was repeated with the lower BSA concentration of 0.04 g/100 cc and the results for the two concentrations are shown in Fig. 15. When the B/A values are normalized to the protein concentrations, the two curves are essentially superimposed upon

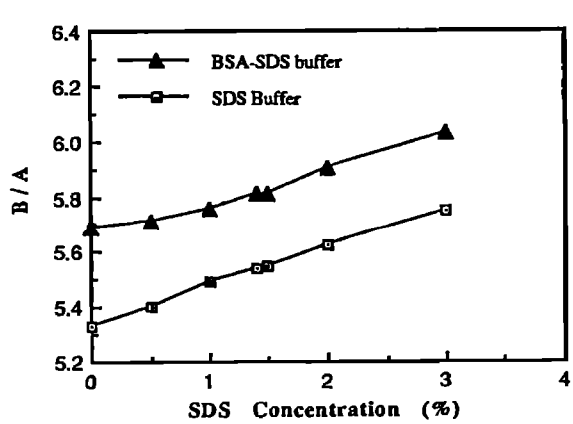

FIG. 13. The B/A values in SDS denaturing buffer with and without the presence of 10% (w/w) BSA protein, exhibiting protein contribution.

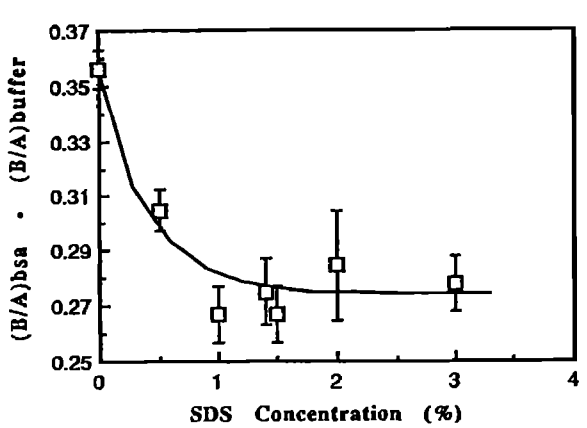

FIG. 14. The difference in B/A values calculated from Fig. 13.

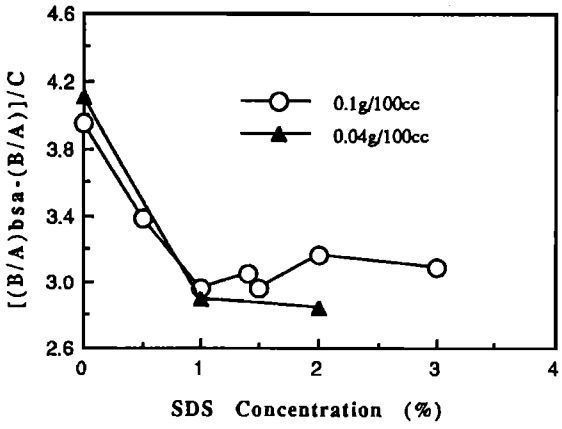

FIG. 15. The B/A difference normalized to the BSA concentration.

each other, demonstrating that the B/A change is independent of the BSA concentration. This result also supports the assumption that the B/A value associated with native secondary structure is large and that with the denatured molecule is reduced.

The conclusion that the B/A value decreases as the BSA protein is denatured should hold irrespective of how the protein is denatured. The hypothesis was tested further by measuring the B/A value in the process of heat denaturation of ribonuclease A. Ribonuclease A is a protein of 14 200 molecular weight and is known to undergo reversible conformational transition between the native and denatured states at about 40 °C in pH 2.77 aqueous solution (Brandts and Hunt, 1967; Brandts, 1965). The B/A was measured in a solution of 3.9% ribonuclease A, pH 2.77. The result is shown in Fig. 16, together with the B/A values of water. The net B/A change due to ribonuclease A itself, shown in Fig. 17, was obtained using the mixture law:

$$(B/A)_{\text{suspension}} = x_{\text{lipid}}(B/A)_{\text{lipid}} + x_{\text{buffer}}(B/A)_{\text{buffer}}, \quad (7)$$

where the x's are the related volume fractions. It is seen that the B/A value due to ribonuclease A decreases as it is denatured, which supports the result obtained by SDS denaturation.

Errabolu *et al.* (1987) have also observed the lower B/A value associated with denatured protein, in studies of the dependence of the B/A parameter on the different constituents of fatty tissues, by decomposing the fat tissues into three components, viz., water, oily residue and solid residue. They pointed out that the solid residue, one of the dehydration products composed largely of denatured proteins, exhibits significantly lower B/A values.

One of the mechanisms suggested to explain different B/A values in aqueous solutions is the bound to free water ratio in the solution (Yoshizumi *et al.*, 1987; Sehgal *et al.*, 1986b). Free water has been shown to have a high B/A value and bound water a low value (Yoshizumi *et al.*, 1987). The B/A value varies as the relative population of free water and bound water changes. Qualitatively, the result in this section can be seen as indirect evidence supporting this hypothesis, since denaturation of protein alters the free and bound water ratio. The amount of bound water associated with a protein increases as the protein is denatured, because a denatured protein takes an extended linear form, and thus has greater surface area. Consequently, an aqueous solution of denatured protein has a greater amount of bound water, which exhibits a smaller B/A value, and a lesser amount of free water, which exhibits a greater B/A value, than that of native protein. Therefore, the effective B/A in the solution of denatured protein exhibits smaller values.

It can also be observed from Fig. 17 that the B/A value for intact protein calculated by Eq. (7), about 13, is close to those of fats (Law *et al.*, 1985; Errabolu *et al.*, 1987) and phospholipids.

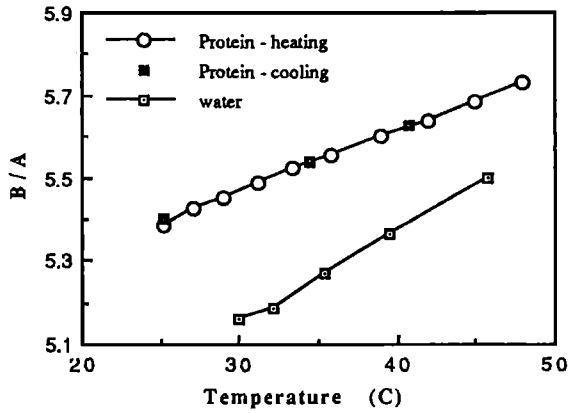

FIG. 16. The B/A in ribonuclease A solution as a function of temperature, during heat and cooling. B/A of water is also shown for comparison.

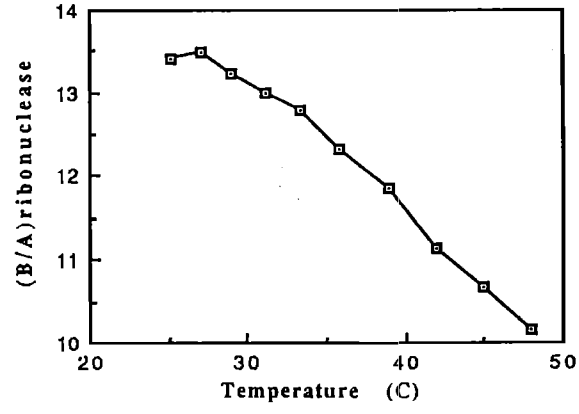

FIG. 17. The B/A contribution from ribonuclease showing decrease as the protein denatured.

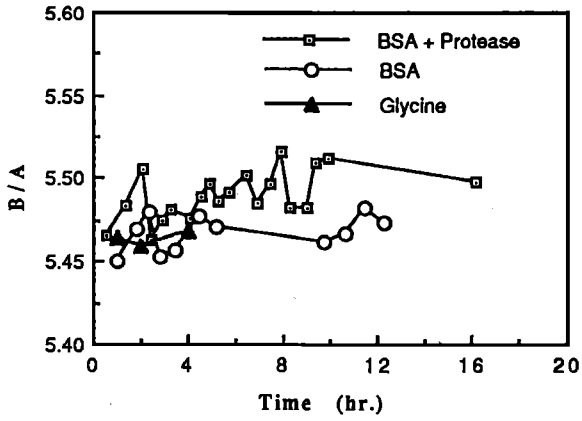

FIG. 18. The B/A in the BSA solution as a function of time, with and without the presence of nonspecific protease.

D. Effect of protein primary structure

To investigate further the dependence of B/A on protein primary structure, a nonspecific protease (Boehringer Mannheim Biochemicals, IN) was used to digest BSA into its constituent amino acids. The underlying idea was to examine the B/A change when this level of protein structure was destroyed. When the nonspecific protease is added to the BSA solution, the enzyme catalyzes the hydrolysis of peptide bonds resulting in the conversion of the protein into free amino acids. For a particular temperature, the higher the concentration of the protease, the faster the rate of digestion. The concentration of protease was chosen so that the entire process of digestion was completed in about 10 h; long enough to collect sufficient data.

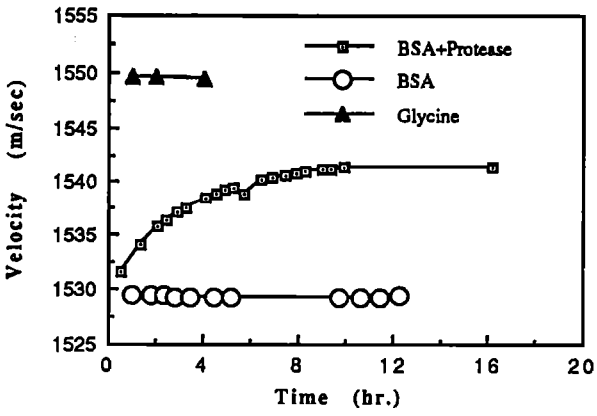

FIG. 19. Speed of sound in the same digestion process.

TABLE II. Relative contributions of three levels of structures.

	Compositional	Structural
Tissue	74%	26%
Cellular	80%	20%
Molecular	85%	15%

The B/A values of BSA aqueous solutions, with and without the presence of protease, are shown in Fig. 18, where it is seen that there is no significant B/A change in the process of digestion, suggesting that the B/A parameter is not sensitive to this level of structure. Also shown in Fig. 18 is B/A for a glycine solution, which exhibits no significant difference in B/A from those in the intact BSA solution and in the digested BSA solution, providing additional evidence for the above conclusion. The speed of sound was also measured in these solutions, shown in Fig. 19, and three observations can be made. First, it reconfirms indirectly the existence of the digestion process, since the only preparation exhibiting the velocity change is the one with protease added. Second, it demonstrates the increase of velocity as the protein is digested. Third, the velocities measured in the glycine solution are greater than both the intact and the digested BSA solutions. These results agree with the conclusion that the digested BSA, which is basically a collection of different amino acids, exhibits higher velocity than intact BSA. The large scattering of the data in Fig. 18 is due to the one time measurement and no attempt is made to average the B/A values, since the 0.5 h per data point is the highest data collection rate the thermodynamic method can achieve.

IV. CONCLUSIONS

The effect of structural change on the B/A parameter has been studied at the tissue, at the cellular and at the molecular levels. At each of these levels, experiments were designed to investigate the contribution to the B/A parameter, due to a particular structural factor by perturbing the structure biochemically, while keeping the remaining B/A influencing factors, such as the chemical composition, unchanged. Specimens employed and the structural factors investigated are considered to be sufficiently representative to enable the conclusion drawn to be generalized.

Two direct conclusions can be drawn from the study: (1) the structural dependance of the B/A parameter exists at all three levels of biological structure, viz., the tissue level, the cellular level and the molecular level; (2) the sensitivity of the B/A parameter to each level of structural change can be arranged in the descending order,

Intercellular adhesion > cellular structure > secondary macromolecular structure > primary macromolecular structure

The relative contributions of compositional and structural factors to the total B/A value of a medium, can be estimated. The relative contributions due to structural features is 26% at the tissue level, 20% at the cellular level, and 15% at the macromolecular level. These are summarized in Table II.

ACKNOWLEDGMENT

This work was supported in part by grants from the National Science Foundation and the National Institutes of Health.

APPPENDIX

Using the notations in Sec. I, Eq. (2) can be rewritten as

$$p_2 = \frac{\pi f(2 + B/A)}{2\rho_0 c_0^3} p_0^2 x e^{-(\alpha_1 + \alpha_2/2)x} \mathrm{DIFF}(x). \quad \text{(A1)}$$

For most biological media, $\alpha_2 \simeq 2\alpha_1$ (Law, 1984), thus

$$p_2 \cong \frac{\pi f(2 + B/A)}{2\rho_0 c_0^3} p_0^2 x e^{-2\alpha_1 x} \mathrm{DIFF}(x). \quad \text{(A2)}$$

A similar equation can also be written for a reference medium as

$$p_2^* = \frac{\pi f(2 + B/A)^*}{2(\rho_0 c_0^3)^*} (p_0^2)^* x^* e^{-2\alpha^* x^*} \mathrm{DIFF}(x^*), \quad \text{(A3)}$$

where * denotes the reference medium. Dividing Eq. (A2) by Eq. (A3),

$$\frac{p_2}{p_2^*} = \frac{(2 + B/A)}{(2 + B/A)^*} \frac{(\rho_0 c_0^3)^*}{\rho_0 c_0^3} \frac{p_0^2}{(p_0^2)^*} \frac{x\,\mathrm{DIFF}(x)}{x^*\,\mathrm{DIFF}(x^*)} \times e^{-2(\alpha_1 x - \alpha^* x^*)} \quad \text{(A4)}$$

can be obtained. Finally, assuming $e^{\alpha_1 x} = p_1/p_0$ and substituting the definitions of η_0, η_1, and η_2 into Eq. (A4), Eq. (3) can be obtained.

Apfel, R. E. (1983). "Effective nonlinearity parameter for immiscible liquids mixtures," J. Acoust. Soc. Am. 74, 1866–1868.

Apfel, R. E. (1986). "Prediction of tissue composition from ultrasonic measurements and mixture rules," J. Acoust. Soc. Am. 79, 148–152.

Beyer, R. T. (1974). Nonlinear Acoustics (Naval Ship Systems Command, New London, CT), p. 107.

Brandts, J. F. (1965). "The nature of the complexities in the ribonuclease conformational transition and the implications regarding clathrating," J. Am. Chem. Soc. 87, 2759.

Brandts, J. F., and Hunt, L. (1967). "The thermodynamics of protein denaturation. III. The denaturation of ribonuclease in water and in aqueous urea and aqueous ethanol mixtures," J. Am. Chem. Soc. 89, 4826.

Cobb, W. N. (1982). "Measurement of the acoustic nonlinear parameter for biological media," Ph. D. dissertation, Yale University, New Haven, CT, pp. 74 and 83.

Drochmans, P., Wanson, J.-C., and Mosselmans, R. (1975). "Isolation and subfractionation on Ficoll gradients of adult rat hepatocytes," J. Cell Biol. 66, 1–22.

Dunn, F., Law, W. K., and Frizzell, L. A. (1981). "Nonlinear ultrasonic wave propagation in biological materials," Proc. IEEE Ultrason. Symp. 527–532.

Errabolu, R. L., Sehgal, C. M., and Greenleaf, J. F. (1987). "Dependence of ultrasonic nonlinearity parameter B/A on fat," Ultrason. Imag. 9, 180–194.

Everbach, E. C. (1989). "Tissue composition determination via measurement of the acoustic nonlinearity parameter," Ph.D. dissertation, Yale University, New Haven, CT.

Gong, X., Feng, R., Zhu, C., and Shi, T. (1984). "Ultrasonic investigation of the nonlinearity parameter B/A in biological media," J. Acoust. Soc. Am. 76, 949–950.

Kawaguchi, F., Kuhlenschmidt, M., Roseman, S., and Lee, Y. C. (1981). "Differential uptake of D-galactosyl- and D-glucosyl neoglycoproteins by isolated rat hepatocytes," J. Biol. Chem. 256, 2230–2234.

Kuhlenschmidt, M. S., Schmell, E., Slife, C. W., Kuhlenschmidt, T. B., Sieber, F., Lee, Y. C., and Roseman, S. (1982). "Studies on intercellular adhesion of rat and chicken hepatocytes: Conditions affecting cell-specificity," J. Biol. Chem. 257, 3157.

Law, W. K. (1984). " Measurement of the nonlinearity parameter B/A in biological materials using the finite amplitude and thermodynamic method," Ph.D. dissertation, University of Illinois at Urbana–Champaign, Urbana, IL, p. 67.

Law, W. K., Frizzell, L. A., and Dunn, F. (1981). "Nonlinear ultrasonic propagation in biological media," J. Acoust. Soc. Am. 69, 1210–1212.

Law, W. K. Frizzell, L. A., and Dunn, F. (1985). "Determination of the nonlinearity parameter B/A of biological media," Ultrasound Med. Biol. 11, 307–318.

Obrink, B., Kuhlenschmidt, M. S., and Roseman, S. (1977). "Adhesive specificity of juvenile rat and chicken liver cells and membranes," Proc. Natl. Acad. Sci. U.S.A. 74, 1077–1081.

Rafferty, J., and Norling, R. (1986). Cricket Graph Software Manual (Cricket Software, Malvern, PA), pp. 6–16.

Seglen, P. O. (1973). "Preparation of rat liver cells, III. Enzymatic requirements for tissue dispersion," Exp. Cell. Res. 82, 391.

Seglen, P. O. (1976). " Preparation of isolated rat liver cells," Methods Cell Biol. 13, 29–83.

Sehgal, C. M., Bahn, R. C., and Greenleaf, J. F. (1984). "Measurement of the acoustic nonlinearity parameter B/A in human tissues by a thermodynamic method," J. Acoust. Soc. Am. 76, 1023–1029.

Sehgal, C. M., Brown, G. M., Bahn, R. C., and Greenleaf, J. F. (1986). "Measurement and use of acoustic nonlinearity and sound speed to estimate compositon of excised livers," Ultrasound Med. Biol. 12, 865–874.

Sehgal, C. M., Porter, B. R., and Greenleaf, J. F. (1986b). "Ultrasonic nonlinear parameters and sound speed of alcohol–water mixtures," J. Acoust. Soc. Am. 79, 566–570.

Spiro, R. (1965). " The carbohydrate units of thyroglobulin," J. Biol. Chem., 240, 1603–1610.

Weber, K., and Osborn, M. (1969). "The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis," J. Biol. Chem. 244, 4406.

Yoshizumi, K., Sato, T., and Ichida, N. (1987). "A physio-chemical evaluation of nonlinear parameter B/A for media predominantly composed of water," J. Acoust. Soc. Am. 82, 302–305.

Zhang, J. (1990) "Influences of biological structural features on the acoustic nonlinearity parameter B/A," Ph.D. dissertation, University of Illinois at Urbana–Champaign, Urbana, IL.

Zhang, J., and Dunn, F. (1987). "*In vivo* B/A determination in a mammalian organ," J. Acoust. Soc. Am. 81, 1635–1637.

Zhang, J., and Dunn, F. (1991). "A small volume thermodynamic system for B/A measurement," J. Acoust. Soc. Am. 89, 73–79.

Zhang, J., Magin, R. L., Frizzell, L. A., and Dunn, F. (1988). "Dependence of the ultrasonic nonlinearity parameter B/A on cellular-level structure," Proc. IEEE Ultrason. Symp. 2, 975–978.