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Differential sound velocity apparatus for the investigation of protein solutions

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A differential method for the measurement of sound velocity in solutions is described. The instrument uses two identical acoustic resonators and a newly developed electronic measuring system. The sound velocity is determined with a relative accuracy of $\approx 3 \times 10^{-6}$ over the entire frequency range 0.5–10 MHz. The instrument has been used to determine the compressibility of protein solutions and to study their velocity dispersion.

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INTRODUCTION

Sound velocity studies have been widely applied to gases, solids, liquids, and solutions. One quantity which has been obtained from these studies is the adiabatic compressibility β_{ad} , which is related to the density ρ and sound velocity c by

$$\beta_{\text{ad}} = -\frac{1}{V} \left(\frac{\partial V}{\partial P} \right)_S = \frac{1}{\rho c^2},$$

where V is the volume, P the pressure, and the subscript S indicates adiabatic conditions. Through measurements of dispersion in the sound velocity, relaxation phenomena have also been detected. Although such sound velocity measurements can be fruitfully applied to biomolecules as well, very few reports have appeared.^{1–8} In the present article we describe an instrument to accurately measure sound velocity in solutions. The apparent adiabatic compressibility of several globular proteins has been determined using this newly developed ultrasonic technique. Measurements were made in the frequency range of 0.5–10 MHz and at different pH values.⁹ The compressibility of the protein interior and its relative contribution to the overall compressibility of the hydrated protein is calculated using a simple model which takes into account the existence of different species of bound water. The results suggest that the protein interior is quite incompressible.

Several methods have been described to measure sound velocity in liquids using either pulse or continuous sinusoidal excitation.^{10,11} Generally for very high frequency, pulse methods are preferred. In the frequency range 0.5–30 MHz, however, the sound velocity can be determined to great accuracy using acoustic resonators. The resonance frequencies (when standing waves are excited in the ultrasonic tank) are often measured near the natural frequency of the quartz transducers, where the efficiency of the resonator is maximum. Different methods have been described to detect the resonance.^{12–19} A common approach is to continuously vary the frequency at the transmitter using a tracking generator and measuring the voltage at the receiver crystal with a spectrum analyzer. At resonance, a sharp increase in the receiving voltage is detected. The width of the resonance peak is typically on the order of 300–500 Hz at 3 MHz in water. This spectrum analyzer method has recently been automated.¹⁹

In order to achieve a relative accuracy of 10^{-6} in sound velocity, the position of the resonance peak must be determined to within 3 Hz at 3 MHz. This accuracy is necessary if dispersion in water solutions is to be investigated.²⁰ The temperature stability of the ultrasonic tank plays a crucial role. For water at 20° C the temperature coefficient of sound velocity is approximately $+2.4 \text{ m/s}^\circ \text{C}$, the sound velocity being on the order of 1500 m/s.²¹ If an accuracy of 10^{-6} is needed, then the temperature stability must be on the order of 10^{-3} K for the duration of the experiment. If the thermal stability is not this good, then the temperature can be monitored and corrections applied. Alternatively, differential methods can be used.³ This temperature effect must be taken into account in the design of the ultrasonic system, and a fast determination of the resonance frequency is necessary if the frequency dependence of the sound velocity is to be studied.

An alternative method to the frequency sweeping has been described for a measurement of a single resonance frequency.¹⁶ This method uses a positive feedback loop in which the signal from the receiver is amplified and sent to the transmitter. This technique offers the advantage of a very accurate and fast determination of the resonance frequency. Our apparatus uses a positive feedback loop but allows the determination of all resonance peaks within a certain frequency range, and does not suffer from the problems due to the nonlinearity of the filter discussed by Reshetnik.

I. ACOUSTIC RESONATOR

The sound velocity is measured using an acoustic resonator. The resonator is similar to the one described by Eggers *et al.*^{22,23} It is composed of a cylindrical tank with a 10-MHz, 1-in.-diam quartz transducer (Valpey-Fisher) at either end. Parallelism of the transducers is necessary to achieve a pure resonance, and is obtained by tuning the three micrometers illustrated in Fig. 1. Figure 2 shows the dimensions of the tank containing the liquid. The tank is made from a 0.015-in.-thick stainless-steel tube, with two O rings at the ends to seal in the liquid. All metal surfaces in contact with the liquid are gold plated. The resonator is tuned using a spectrum analyzer with tracking generator plug-in (Tektronix model 7L5 with option 25). The output from the gen-

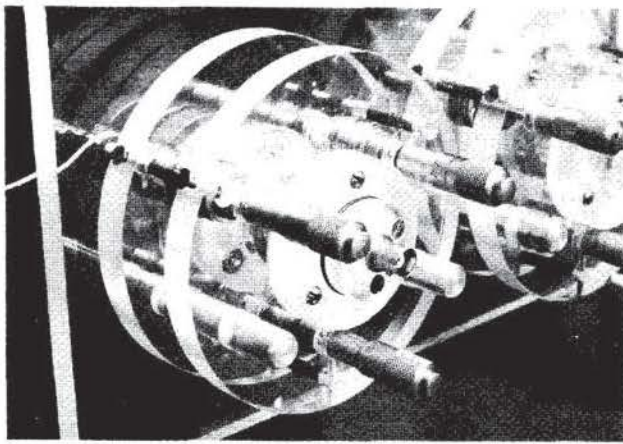


FIG. 1. The ultrasonic tank.

erator is applied to one crystal and the signal at the other crystal is analyzed. A typical spectrum is shown in Fig. 3. The peaks correspond to standing wave formation, i.e., when the length L of the tank is a multiple of half a wavelength

$$L = n \frac{\lambda}{2},$$

or the equivalent relation,

$$f_n = \frac{n}{2L}c.$$

If n and L are known, sound velocity c can be obtained by measuring the frequency for standing wave formation.

There are a large number of resonance peaks at intervals of approximately 60 kHz, depending on the length of the tank. The useful range of the resonator extends from about 500 kHz to 30 MHz. Once the tank has been tuned, i.e., when each resonance peak for the entire frequency range appears as a single peak with no shoulders (Fig. 4), the spectrum analyzer is replaced by an electronic system to accurately measure the resonant frequency of some of the peaks. The electronic system is essentially an oscillator circuit with the ultrasonic tank in the positive feedback (Fig. 5). The portion

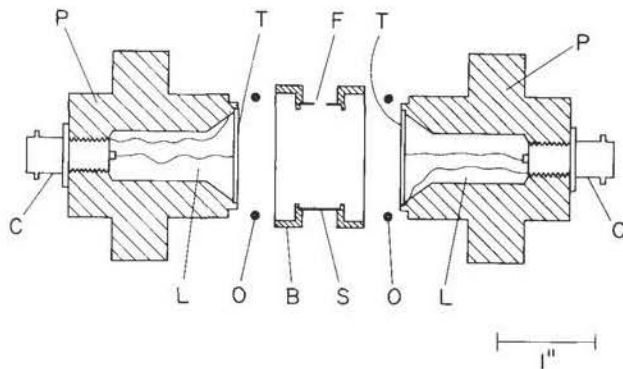


FIG. 2. Cross section of the inner part of the ultrasonic tank. Micrometer adjustment mechanism is not shown. B: Brass flange; C: BNC connectors; F: filling hole; L: leads for electrical contact; O: O rings; P: Plexiglas mount; S: stainless-steel cylindrical tube.

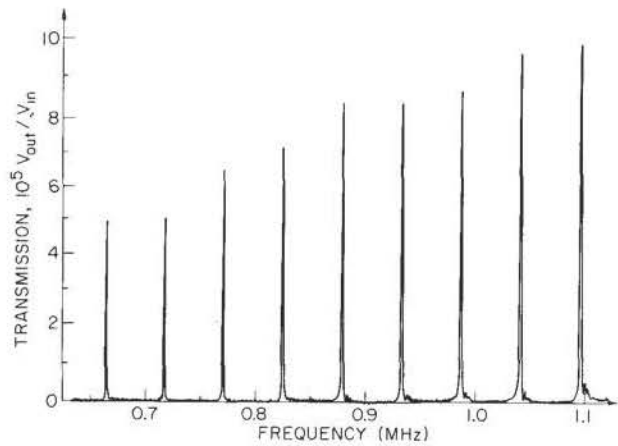


FIG. 3. Portion of the frequency spectrum of the ultrasonic tank.

of the circuit enclosed in the dashed box operates as a frequency selective amplifier. A nonvanishing steady-state signal is attained if the gain and phase of the signal around the loop satisfy the condition for stable oscillation. This condition depends on the transmission of the tank T . The phase shift introduced by the amplifiers, mixers and filter is slowly varying over the entire frequency range and can be neglected because of our differential method (see below). The phase constancy is achieved by using wideband amplifiers A1 and A3 and by keeping the output of the low-pass filter A2 always at the same frequency. The amplitude and phase response of the tank are shown in Fig. 6 for one of the even-numbered resonance peaks. The phase shift is zero at the maximum of transmission (for odd peaks the phase shift is 180° at the maximum). In our oscillator circuit the amplitude

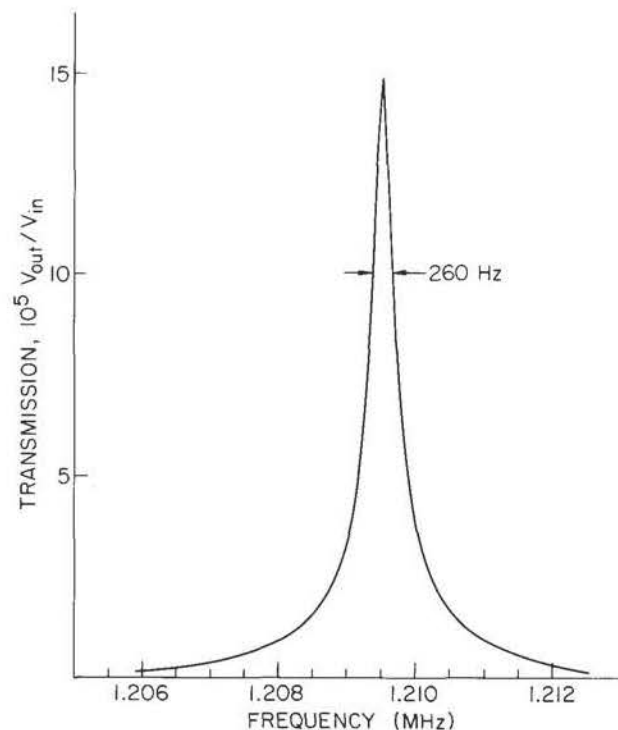


FIG. 4. Expanded view of a single resonance peak.

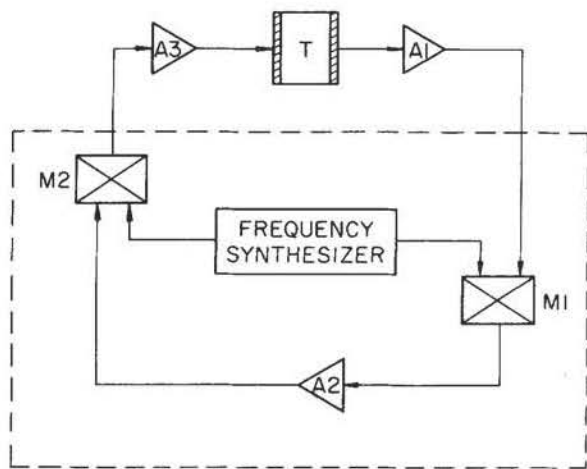


FIG. 5. Block diagram of the electronic loop. T: Ultrasonic tank; A1: wide-band amplifier (Tektronix 1121); A2: filter amplifier (PAR 113); A3: wide-band amplifier (Power Labs A102); M1: mixer (Mini-Circuits ZAD 1); M2: mixer (Mini-Circuits ZAD 6). The frequency Synthesizer is a Hewlett-Packard 3325A.

of the signal is limited by the saturation of mixer M2. The gain of the low-pass filter amplifier is varied according to the sound absorption in the tank in order to keep the saturation level of the mixer at a constant value. This procedure ensures a constant electronic phase delay. The above configuration works quite well for the weakly absorbing solutions used in our studies. We have not tested the apparatus with strongly absorbing liquids. Although the transmission of the tank varies by more than a factor of 100 from 500 KHz to 10 MHz, the oscillation condition can be easily maintained. This means that the attenuation due to strongly absorbing materials can be compensated for in our system by increasing the gain of the amplifiers.

In order to start the oscillation on a desired resonance of the tank, a synthesizer (Hewlett-Packard model 3325A) generates a fixed frequency f_c near the resonance. The output of the synthesizer is connected to two mixers (Mini-circuits models ZAD1 and ZAD6). The ultrasonic tank acts as

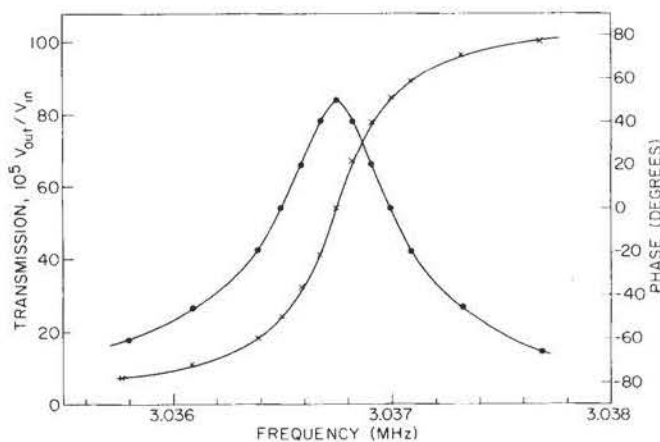


FIG. 6. Amplitude and phase response for a resonance peak.

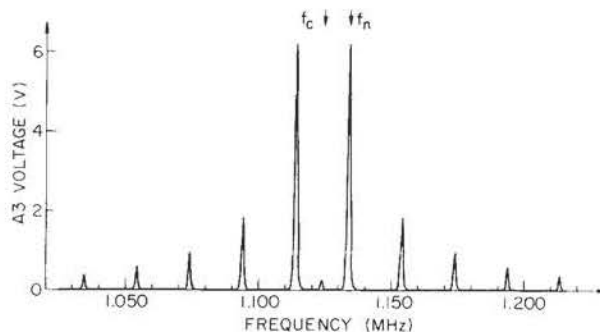


FIG. 7. Frequency spectrum at the output of mixer M2.

a very narrow filter, which transmits only a particular resonance frequency f_n . Amplifier A1 is a wideband amplifier (Tektronix model 1121). At the output of mixer M1 frequencies $f_n \pm f_c$ are present. Suppose f_c and f_n are on the order of 1 MHz. The filter amplifier A2 (Princeton Applied Research, model 113) is a low-pass filter with 20-KHz bandwidth and selects $f_n - f_c$. At the output of mixer M2 two frequencies are available: $f_c \pm (f_n - f_c)$. In Fig. 7 the spectral analysis of the signal at the output of mixer M2 is shown. The harmonic content is caused by the saturation of the mixer. This signal is amplified by the wideband amplifier A3 (Power Labs model A102). Because of the filtering action of the tank, only f_n is available at the input of amplifier A1. Figure 8 shows the spectral analysis after the tank. If the gains of amplifiers A1, A2, and A3 are sufficiently high the system will resonate at f_n . The frequency at the output of amplifier A2 is kept constant to within 10 Hz by manually adjusting f_c . This is done in order to minimize the effect of the phase shift due to the low pass filtering, as stated above. The exact value of the resonance frequency is obtained by measuring the output of A2 with a frequency meter (Hewlett-Packard model 5328A) and adding this frequency to f_c , which is displayed on the frequency synthesizer readout. A different resonance peak is selected by changing f_c . Note that the circuit will oscillate only if f_c is within 20 kHz of one of the resonance frequencies of the tank. This is easily achieved by varying f_c continuously until resonance is detected by an oscilloscope connected at the output of A2. The resonance frequency is measured with a resolution of 1 Hz over the entire range.

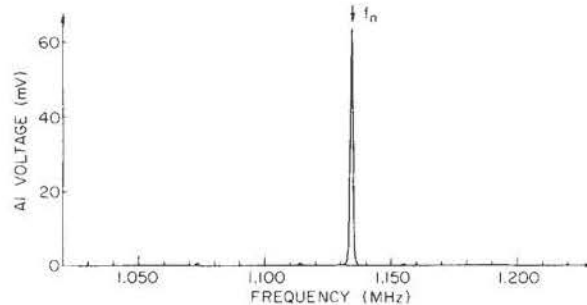


FIG. 8. Frequency spectrum at the output of the tank.

II. MEASUREMENTS

We are interested only in the difference between the sound velocity in the solution c_p and in the solvent alone c_s . We use two nearly identical resonators to correct for temperature fluctuations. These are enclosed in a thermostated chamber and the temperature controlled to 0.02 K during the measurements. In order to obtain the difference in sound velocity between the solvent and the solution we perform a series of three measurements. The reference cell is filled with distilled water and is used essentially as a very sensitive thermometer. The sample cell is first also filled with distilled water and the resonance frequencies of the two cells measured. The ratio of the two frequencies for the same resonance order n is equal to the ratio of the length of the two cells. Then the sample cell is filled with solvent and the resonance frequencies of the two cells are again measured for the same n . Finally the sample cell is filled with the solution and the two resonance frequencies are measured. The difference between sound velocity in the solution and in the solvent alone is obtained as follows:

$$c_p - c_s = \left[\left(\frac{f_p}{f_{3w}} \right) - \left(\frac{f_s}{f_{2w}} \right) \frac{f_{1w}}{f_w} \right] c_w.$$

In Table I a typical measurement is reported along with the meaning of the symbols used above. The differential method compensates for three things: the small differences in the construction of the two resonators, the small temperature variations between two consecutive measurements, and the small phase shift introduced by the measuring circuit. The ratio of the frequencies in the two cells is stable over a long period of time and is reproducible to within 2×10^{-6} . Generally eight resonance peaks are measured equally spaced on a logarithmic frequency scale. A complete set of measurements takes about 10 min.

For small volume fractions v of the solute, the relative excess sound velocity $1/v(c_p - c_s/c_s)$ may be defined, where c_p and c_s are the sound velocity of the solution and solvent respectively. The apparent compressibility β_1 of the solute is related to this quantity by

$$\frac{\beta_1}{\beta_0} = 2 - \frac{\rho_1}{\rho_0} - \frac{2}{v} \left(\frac{c_p - c_s}{c_s} \right),$$

where β_0 and ρ_0 are the compressibility and density, respectively, of the solvent and ρ_1 is the apparent density of the solute. In our studies the relative excess sound velocity is determined with an accuracy of 10^{-2} for protein concentrations in the range 0.5%–2% in volume. Figure 9 shows a plot of the ratio of the solution and solvent compressibilities β/β_0

TABLE I. Typical sound velocity measurement for one resonance order n .

Measurement number	Sample cell		Reference cell	
	contents	frequency (Hz)	contents	frequency (Hz)
1	water	$f_w = 2\,989\,611$	water	$f_{1w} = 2\,990\,748$
2	solvent	$f_s = 2\,997\,301$	water	$f_{2w} = 2\,990\,532$
3	solution	$f_p = 3\,003\,868$	water	$f_{3w} = 2\,990\,549$

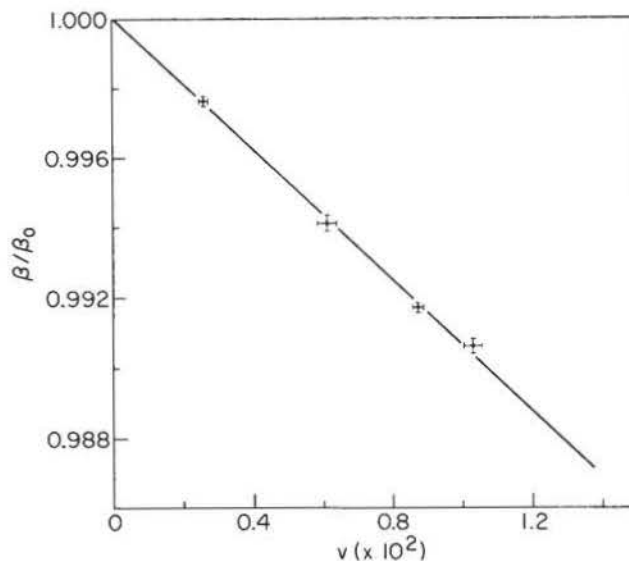


FIG. 9. Solution compressibility relative to water as a function of the volume fraction of a myoglobin solution at 25° and pH 7.2. ($\beta_0 = 4.5 \times 10^{-10} \text{ m}^2/\text{N}$).

vs v for myoglobin. The plot is linear, indicating ideal behavior for the range of concentration studied.⁹ The error in the figure corresponds to the standard deviation of a series of measurements. The error is determined by the reproducibility of the protein preparation rather than by the accuracy in the determination of sound velocity.⁹

We have used the above technique to measure dispersion in the sound velocity for protein solutions and amino acid solutions at high pH. Figure 10 shows the excess sound velocity relative to the solvent as a function of frequency for a 580- μM solution of myoglobin at pH 11.7 and a 100-mM glycine solution at pH 11.6. The oscillation of the points

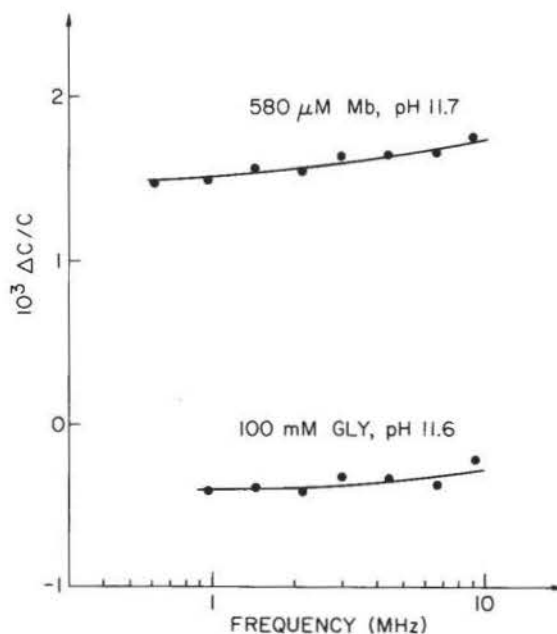


FIG. 10. Excess sound velocity as a function of frequency for glycine and myoglobin at 25°.

around the expected line is due neither to random error nor to temperature fluctuations, but is instead systematic, reproducible, and dependent on the tuning of the tank. The dispersion in Fig. 10 is caused by proton transfer processes between the solvent and those accessible charged groups whose pK's are near the pH of the solution.^{24,25} These data are consistent with the amplitudes and frequencies of dispersion predicted from sound absorption measurements on similar systems.^{1,25-29}

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