Consequently, Bertaut's method for evaluating the Coulombic lattice energy in a crystal can be usefully extended to application in lattice dynamics, and in a 'natural' way it implicitly accounts for a remarkably detailed treatment of the problem.

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# Thermal Motion in Protein Crystals Estimated Using Laser-Generated Ultrasound and Young's Modulus Measurements


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## Abstract

The measurement of the longitudinal speed of sound in crystals of ribonuclease-A and in human haemoglobin using laser-generated ultrasound is reported. Average values of 1784 (72) m s$^{-1}$ and 1828 m s$^{-1}$ were obtained. As a control the speed of sound transmitted through a compacted disc of lysozyme powder was measured as 2004 (23) m s$^{-1}$. The measured longitudinal acoustic velocities and the transverse velocity, estimated from a knowledge of Young's modulus, were used to estimate the acoustic contribution to the mean-square displacements of protein molecules as determined by X-ray crystallography. It was found that thermally induced acoustic vibrations make a significant contribution to the total atomic disorder, estimated to be in the range 0·04–0·11 Å$^2$ for the proteins studied. Such single-crystal estimates are required for calculation of the acoustic component of the diffuse scattering in protein crystal X-ray diffraction.


## Introduction

X-ray diffraction from protein crystals yields patterns comprised of two components, namely, the Bragg and the diffuse scattering. The measurement of the diffuse scattering and attempts at its interpretation are a new development. The advent of area detectors, high-intensity synchrotron radiation and supercomputers means that it is now easier to try to exploit the


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complete diffraction pattern. This should lead to improved insights into protein structure and mobility.

A well known feature of many macromolecular X-ray diffraction photographs is the occurrence of diffuse halos surrounding the Bragg reflections. Diffraction theory accounts for these in terms of propagation of acoustic vibrations in crystals and this aspect of thermal diffuse scattering has been extensively studied in crystals of small molecules (Criado, Conde & Márquez, 1985). These vibrations contribute to the atomic motion in crystals and it is interesting to investigate the proportion of the mean-square atomic displacements that can be attributed to acoustic effects.

Mean-square displacement amplitudes (MSDA) of atoms are commonly obtained from refinements of crystal structures obtained from X-ray diffraction. But such experiments do not readily distinguish between the various contributions to the MSDA which may be local diffuse motions, rigid-body movements of molecules or their domains or else static disorder within the crystal. These different types of motion have been reviewed by Stuart & Phillips (1985). Mössbauer spectroscopy and experiments at different temperatures (Frauenfelder, Petsko & Tsernoglou, 1979) have helped to identify the static component but do not address the contribution due to thermally induced ultrasonic vibrations. Mössbauer diffraction has been used to estimate the dynamics of the protein myoglobin; a r.m.s. displacement of 0·13 Å was attributed to intermolecular motions on time scales shorter than 100 ns (Nienhaus, Heinzl, Huenges & Parak, 1989). Doster, Cusack & Petry (1989) have used incoherent inelastic neutron scattering to investigate atomic motion in hydrated myoglobin over the temperature range 4–350 K and on the molecular dynamic time scales of 0·1–100 ps; at 180 K the mean-square hydrogen displacement is ~0·02 Å$^2$ and at 300 K it is 0·17 Å$^2$. A review of neutron techniques applied to protein dynamics can be found in the book edited by Schoenborn (1984).

The mean-square molecular displacements caused by acoustic waves in crystals may be derived from a knowledge of the quasi-transverse and quasi-longitudinal velocities of sound in the crystals. The longitudinal velocity and its associated modulus may be measured by the methods indicated below and results are reported in this paper. The transverse velocity was, however, not measurable with protein crystals for reasons given later. However, the transverse velocity can be calculated from the longitudinal modulus and Young's modulus, the latter having already been measured for a range of protein crystals (Morozov & Morozova, 1981).

The measurement of the velocity of sound in crystals of graphite has been made by Willis, Carlile, Ward, David & Johnson (1986) using pulsed neutron diffraction. The advantage of neutron scattering is that the sample is easily aligned to present specific directions to the beam by goniometry. The disadvantage of the method, in terms of its application to other crystal systems, is the need for large crystals and a relatively small unit cell.

Brillouin scattering has also been used for measuring sound velocities in materials of biological significance (Vaughan & Randall, 1982; Vaughan, 1981). Accuracies of a few percent are attainable from specimens with suitable optical properties.

An alternative method for measuring the speed of sound is by use of ultrasonics (Scruby, Dewshurst, Hutchins & Palmer, 1982). This is the method we have used. The requirement for a small unit cell is relaxed completely compared with the neutron method. However, a large cross section and relatively thick crystal is required; the latter is a fundamental limit to the accuracy obtainable, although short-duration ultrasonic pulses and accurate timing techniques will improve the situation. The former requirement could be eased with improved alignment of the apparatus when the ultrasound is laser generated.

## Experimental method

The speed of sound in a material was measured ultrasonically *via* two different approaches. Namely, the sound wave was generated by a piezoelectric transducer or induced by a laser pulse. In both methods the echo was registered by a transducer.

The laser-induced sound wave method was used for all of the measurements on the protein single crystals. The crystals used were ribonuclease-A and haemoglobin. The crystals were representative of those used in each case to reveal the protein structures at high resolution.

The ribonuclease-A crystals are composed of 50% solvent of which 30% is ethanol and 70% is water. The crystallographic parameters are space group $P2_1$, cell dimensions $a = 30{\cdot}45$, $b = 38{\cdot}37$, $c = 53{\cdot}22$ Å, $\beta = 106{\cdot}0°$. Two crystals were used in the ultrasound measurements. One measured $7{\cdot}7 \times 6{\cdot}2 \times 2{\cdot}7$ and the other $3{\cdot}0 \times 2{\cdot}625 \times 1{\cdot}875$ mm. The face presented to the beam for the first crystal was 2·7 mm thick and uniform to better than 0·1 mm. For the second crystal two orientations were used of thicknesses 1·875 and 2·625 mm.

The haemoglobin used was of human origin and the crystals are composed of 50% solvent (PEG and water). The crystallographic parameters (for the methaemoglobin) are space group $P2_12_12$, cell dimensions $a = 95{\cdot}8$, $b = 97{\cdot}8$, $c = 65{\cdot}5$ A. One crystal was used of dimensions $4{\cdot}9 \times 2$ mm with the third dimension varying from 1·68 mm at its thinnest to 2·22 mm at its thickest. The thickness of the crystal at its centre is 1·95 mm and over the width of the laser-beam diameter (~0·5 mm) this is almost constant.

A control sample was used which comprised of lyophilized lysozyme powder. This was compacted into a dense disc of uniform thickness 4·6 mm and diameter of 30 mm. Measurements on this sample were *via* both the laser and piezoelectric (direct-contact) methods.

The choice of materials was made so as to give some idea of the range of the measured values for two different protein crystals and four crystal orientations. The lyophilized lysozyme disc provides an upper limit for the speed of sound $v_l$ and the tabulated value of water (1400 m s$^{-1}$) a lower limit. All our experiments were conducted at room temperature.

A schematic of the experimental arrangement is shown in Fig. 1. For the sample cell there appeared, at first, to be two conflicting requirements. Firstly, the need to keep the crystal bathed in its mother liquor and, secondly, the need to provide a surface for laser generation of ultrasound to occur and to ensure good acoustic coupling between the crystal and both this surface and the receiving transducer. The crystal was placed in a well in an aluminium block and bathed in mother liquor nearly to the level of the uppermost surface of the crystal. A small aluminium disc, of known thickness, was placed on top of the crystal. The receiving transducer supported the aluminium block. An Nd:YAG (yttrium aluminium garnet) laser pulse ($\lambda$ 1·064 μm, 15 ns duration with 20 mJ of energy per pulse) was focused onto the aluminium disc to produce an ablation source and this generated the sound wave through the crystal. Ultrasonic waveforms were detected by a 5·0 (5) MHz well damped Panametrics PZT transducer and captured on a Tektronix 7912 AD 9-bit digitizer. The digitizer was integrated into a Tektronix MS4101 minicomputer system based on a PDP-11/34 on which signal processing and interpulse timing were carried out. In the main only the direct ultrasonic pulse was observable and timings were made from the initiating laser pulse to the first ultrasonic arrival. The contribution of aluminium to the time delay is allowed for; in no experimental run did this account for more than 1/6 of the measured time of 1-2 μs. Fig. 2 shows an example of the captured ultrasonic trace. The laser generated a broad bandwidth pulse with frequency components extending from below 1 MHz to above 10 MHz.

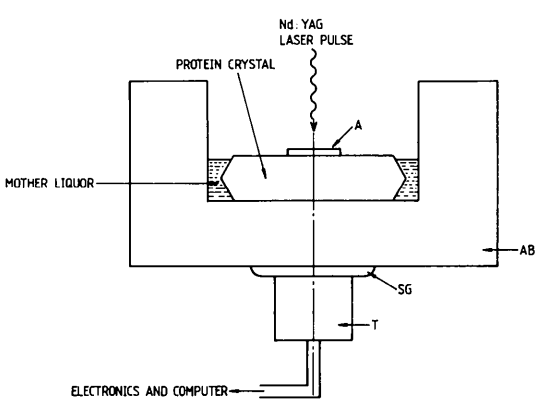


Fig. 1. A schematic diagram of the experimental arrangement to measure the speed of sound by laser pulse generated ultrasound. A = aluminium disc, AB = aluminium block, SG = silicone grease, T = transducer.

## Experimental results

The results are summarized in Table 1. The values obtained for the various crystals/crystal settings all lie between the extremes set by the lyophilized lysozyme disc and water. The standard deviation on the value for the disc is very good and both methods (piezo *vs* laser) agree very well. The deviation of the values from the mean for the different crystals and propagation directions is not significant. Much of this dispersion occurs within measurement set (3) (*i.e.* values of 1819, 1883 and 1717 m s$^{-1}$) where repeated attempts were made in order to assess the effectiveness of the alignment of the laser beam onto the crystal and the repeatability of the ultrasonic traces. It is therefore not possible to reveal the anisotropy of the speed in different directions except to say that it is probably less than one standard deviation (67) in 1791 m s$^{-1}$ (*i.e.* $<4\%$). Improvements in the laser alignment onto the crystal and its relative orientation with respect to the crystal axes would require a more sophisticated experimental arrangement to be constructed. It is important to note that although shear (transverse) waves were generated, only one broad shear wave was observed making accurate timing difficult. Hence, no reliable estimate of the transverse velocity was possible with this method for a protein crystal.

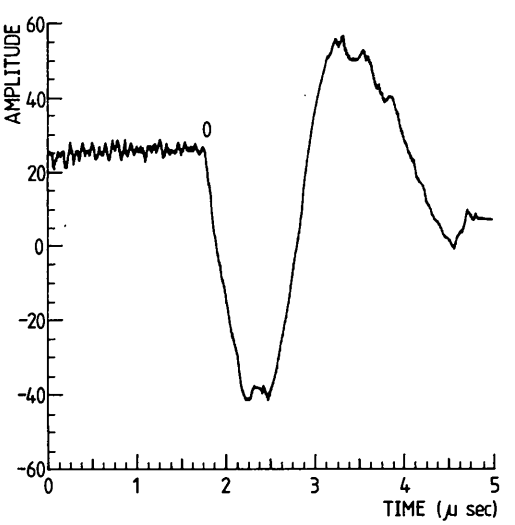


Fig. 2. An example of one of the ultrasonic traces.

Table 1. *Measured values of the speed of sound*

| Material | Method | Speed ($m\ s^{-1}$) |
|---|---|---|
| 1. Lysozyme compacted disc | Laser | 1985 |
| | Laser | 2029 } av. = 2004(23) |
| | Piezo | 1999 |
| 2. Ribonuclease crystal 1 | Laser | 1712 |
| 3. Ribunuclease crystal 2 (orientation 1) | Laser | 1819 |
| | | 1883 } av. = 1806(84) } av. = 1784(72) } av. = 1791(67) |
| | | 1717 |
| 4. Ribonuclease crystal 2 (orientation 2) | Laser | 1789 |
| 5. Haemoglobin | Laser | 1828 |

Notes: 1. The speed of sound in water at 293 K is 1470 $m\ s^{-1}$. 2. The speed of sound in 20% ethanol/80% water at 293 K is 1580 $m\ s^{-1}$. 3. All above measurements were made at room temperature (~293 K). 4. The average speeds calculated in the table were arrived at in the following way. The value of 1806 $m\ s^{-1}$ is an average over 1819, 1883 and 1717; that of 1784 is from 1712, 1819, 1883, 1717 and 1789; that of 1791 is from 1712, 1819, 1883, 1717, 1789 and 1828 $m\ s^{-1}$.

## Theory and interpretation – atomic displacement from elastic moduli

The ultrasound pulses set up quasi-longitudinal waves in the crystal. The measured speed of sound $v_l$ for the longitudinal modes in the crystal is related to the longitudinal modulus $M$ by the relation

$$v_l = (M/\rho)^{1/2} \qquad (1)$$

where $\rho$ is the density of the crystal.

Using the average $v_l$ value for ribonuclease-A crystals of 1784 (72) $m\ s^{-1}$ (see Table 1) and the density of wet ribonuclease-A crystals $\rho = 1{\cdot}22\ Mg\ m^{-3}$ (Carlisle & Scouloudi, 1951) gives a value for $M$ of 3·88 (8) GPa.

From measured values of $M$ and $E$, it is possible to derive other elastic moduli and in particular the transverse (or shear) modulus $G$ from which the velocity of transverse acoustic waves may be calculated. The relevant equations (Blitz, 1971) are

$$G = \tfrac{3}{4}(M - K) = E/2(1+\sigma) \qquad (2)$$

and

$$K = E/3(1-2\sigma), \qquad (3)$$

$E$ being Young's modulus, $K$ the bulk modulus and $\sigma$ Poisson's ratio.

Morozov & Morozova (1981) have measured Young's modulus $E$ for crystals of lysozyme, haemoglobin and myoglobin, using the method of transverse resonance vibrations. Their crystal forms do not correspond to those used in our experiments so that we can only use their $E$ values to obtain semiquantitative results. The $E$ values for wet protein crystals were found to be approximately 0·7(4) GPa. With this value of $E$ and our value of $M$, equations (2) and (3) yield approximate estimates of Poisson's ratio $\sigma = 0{\cdot}47$; bulk modulus $K = 3{\cdot}6$ GPa; shear modulus $G = 0{\cdot}24$ GPa.

The speed of sound $v_t$ for transverse modes in the crystal is given by

$$v_t = (G/\rho)^{1/2} \qquad (4)$$

which yields an estimate of $v_t = 442(90)\ m\ s^{-1}$ for ribonuclease-A crystals.

The MSDA of protein molecules due to thermally excited acoustic vibrations may be estimated by summing the contributions from normal modes associated with allowed wave vectors in the first Brillouin zone. We make the assumption that the velocity of sound is independent of wavelength and also that the crystals are approximately isotropic. For an isotropic medium there are three modes of polarization, one longitudinal and two transverse. If the speed of sound is $v_i$ for a particular polarization $i$ then in the Appendix we show that the MSDA of the protein molecules due to modes of this polarization is given by

$$\langle x^2\rangle_i = [k_B T/(2\pi^2 \rho v_i^2)](6\pi^2/V_c)^{1/3} \qquad (5)$$

where $V_c$ is the volume of the unit cell.

For ribonuclease-A, $V_c = 60{\cdot}285 \times 10^{-27}\ m^3$ and this gives $\langle x^2\rangle_l = 0{\cdot}0054\ Å^2$ and $\langle x^2\rangle_t = 0{\cdot}08\ Å^2$ at room temperature.

The transverse contribution to the total mean-square displacement is thus an order of magnitude larger than the longitudinal value. The total mean-square displacement due to the three modes of polarization is given by

$$\begin{aligned}\langle x^2\rangle_T &= \langle x^2\rangle_l + 2\langle x^2\rangle_t \\ &= 0{\cdot}0054 + 0{\cdot}16 \\ &= 0{\cdot}1654\ Å^2. \end{aligned} \qquad (6)$$

This value must be divided by three to yield the projected MSDA for comparison with the projected MSDAs customary in X-ray analysis. Thus we finally obtain an estimate of the acoustic contribution, at room temperature, to the total MSDA of

$$\langle u^2\rangle = 0{\cdot}06(3)\ Å^2.$$

The error range is estimated from the range of values for Young's modulus [0·7(4) GPa] measured by Morozov & Morozova (1981) for lysozyme, haemoglobin and myoglobin. An analogous calculation for the monoclinic haemoglobin of Morozov & Morozova using a value of the longitudinal modulus estimated from our orthorhombic haemoglobin yielded $\langle u^2\rangle$ 0·06(1) $Å^2$. The vibrations that we have studied include time scales longer than 0·2 μs.

Morozov & Morozova (1986) related their measurements of Young's modulus $E$ to the translational thermal motion of protein globules $\langle x^2 \rangle_T$, assuming uncorrelated motion of the globules. In the simplified model used, the protein is regarded as a point mass moving in a square potential well (of length $a$) and linked by springs (with force constant $\kappa_T$) representing the elasticity of the lattice cell. The elasticity of the spring is defined by

$$\kappa_T = Ea. \tag{7}$$

The mean-square deviations of a molecule from its average position along an array of molecules is given by

$$\langle x^2 \rangle_T = k_B T / 2\kappa_T. \tag{8}$$

Hence

$$\langle x^2 \rangle_T = k_B T / 2Ea. \tag{9}$$

For crystals of lysozyme, values for $\langle x^2 \rangle_T$ in the range 0·04 to 0·08 Å$^2$ were obtained. These values are in agreement with the value of $\langle u^2 \rangle$ of 0·06(3) Å$^2$ for ribonuclease-A.

Similar calculations for the other hydrated crystals in Table 1 yield values of $\langle u^2 \rangle$ in the range 0·04 to 0·11 Å$^2$.

## Discussion

Both X-ray diffuse scattering (Wooster, 1962) and Brillouin scattering (Randall & Vaughan, 1979) provide direct evidence for the existence of acoustic vibrations in crystals, and Brillouin scattering provides similar evidence for amorphous solids and liquids. We are currently analysing X-ray diffraction profiles of ribonuclease-A reflections obtained from a highly collimated synchrotron source which show intensity variations expected from an equilibrium distribution of acoustic phonons.

Our velocity of sound measurements are the first to be reported in the literature for protein crystals. They are in broad agreement with several velocity measurements made by Vaughan & Randall (1980) using Brillouin scattering from tissues of similar protein concentrations to that found in our crystals. Thus these authors determined a longitudinal velocity of 1850 m s$^{-1}$ for the nucleus of cow lens which has a density of 1·25 Mg m$^{-3}$.

The estimates of transverse velocities have had to make use of Young's modulus measurements made by Morozov & Morozova (1981) on different crystal forms of lysozyme, myoglobin and haemoglobin. Nevertheless whatever value of Young's modulus is assumed within their observed range (0·4–1·1 GPa), transverse velocities (325–558 m s$^{-1}$) are at least a factor of three smaller than the longitudinal values and the acoustic $\langle u^2 \rangle$ is in the range 0·04–0·11 Å$^2$.

The low value of the transverse (shear) modulus in protein crystals and high Poisson ratio is probably related to the smaller number of intermolecular contacts in such crystals where solvent content typically ranges from 27 to 65% (Matthews, 1968). The intermolecular contacts provide the only resistance to shear stresses which vanish completely in a liquid where the transverse degrees of freedom become diffusive.

Three assumptions have been made in our calculations. Firstly the protein crystals have been assumed to be elastically isotropic. Morozov & Morozova (1981) found very little anisotropy in Young's modulus and we have not found significant anisotropy in the sound velocity measurements reported in this paper. Our own diffuse scattering measurements on ribonuclease-A (unpublished results) also do not show significant anisotropy. A second assumption is that there is no dispersion of the acoustic velocities. This is not likely to be true and will probably cause underestimation of the acoustic mean-square displacements. Thirdly we have assumed that the unit-cell contents move as a rigid body. Acoustic vibrations will consist of rigid-body translations for the longer acoustic waves but for shorter wavelengths some molecular distortion may well take place since the forces between protein molecules are of the same order as those between molecular domains. Librational motion, however, contributes mostly to optic modes which will not be excited at frequencies in the MHz range.

The model of Morozov & Morozova (1986) referred to above assumes independent molecular oscillators and gives values of mean-square displacements similar to those estimated in this work. However, in order to obtain quantitative results, it is likely that a model that takes account of the physics of acoustic vibrations will be necessary.

## Comparison with X-ray results

It is not easy to estimate the contribution of intermolecular vibrations to total MSDA from X-ray diffraction data. Parak, Frolov, Mössbauer & Goldanskii (1981) looked for the smallest value of the MSDA of individual atoms in metmyoglobin. As this was 0 Å$^2$ they considered that a substantial contribution from intermolecular vibrations was unlikely. However, individual atomic MSDAs are highly susceptible to small errors in the X-ray model which are almost inevitable in a protein, particularly in the less well defined side chains. We have refined a translation tensor against the X-ray data from ribonuclease-A, applying the **TLS** model of Schomaker & Trueblood (1968) to the whole protein molecule (Howlin, Moss & Harris, 1989). This tensor is almost isotropic with a mean diagonal element of 0·117 Å$^2$. Like the acoustic estimate of 0·06 (3) Å$^2$, the translation tensor will also contain a contribution from non-rigid-body motion. A significant difference between the two estimates is that the $T$ tensor value includes static

disorder which is absent from the acoustic estimate. More recently, Nienhaus *et al.* (1989) used Mössbauer crystallography to estimate a mean-squared amplitude for intermolecular motions of 0·02 Å$^2$ at room temperature for time scales of 100 ns or less.

## Summary

We have shown how we may estimate the mean-square atomic displacements in protein crystals which are due to thermally induced acoustic vibrations. For more precise estimates, some knowledge of acoustic dispersion would be required which may be supplied from inelastic neutron diffraction experiments. However, even with our semi-quantitative estimates of 0·04–0·11 Å$^2$, it is evident that intermolecular vibrations contribute significantly to atomic disorder in protein crystals.

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## APPENDIX

We assume that in acoustic modes of vibration molecular motion approximates to rigid-body translations. For a unit cell of mass $m$ undergoing acoustic vibration in a crystal containing $N$ unit cells, the mean-square displacement due to normal mode $j$ is given by

$$\langle x_j^2\rangle = k_B T/N\omega_j^2 m \qquad (A1)$$

where $k_B$ is Boltzmann's constant, $T$ is the absolute temperature, $\omega_j$ is the angular frequency and $m$ is the mass of the cell.

If we neglect acoustic dispersion then velocity is independent of frequency and

$$\omega_j = v_j q_j \qquad (A2)$$

where $q_j$ is the magnitude of the wave vector $\mathbf{q}_j$ and $v_j$ is the velocity of sound. There is one allowed vector $\mathbf{q}$ per volume element $8\pi^3/(NV_c)$ in the first Brillouin zone where $V_c$ is the unit-cell volume. If we approximate this zone by a sphere of radius $q_m$ then there are $N$ allowed wavevectors within this sphere. Hence

$$\tfrac{4}{3}\pi q_m^3 = 8\pi^3 N/(NV_c) = 8\pi^3/V_c.$$

Hence

$$q_m = (6\pi^2/V_c)^{1/3} \qquad (A3)$$

If the density of states is $g(q)$, then the sum of the mean-square displacements for one acoustic branch $i$ in the Brillouin zone can be found by integrating over the sphere, and

$$\langle x_i^2\rangle = \sum_j \langle x_j^2\rangle = \int_0^{q_m} g(q)\langle x^2\rangle \, dq.$$

However,

$$g(q) = 4\pi q^2 NV_c/8\pi^3 = NV_c q^2/2\pi^2. \qquad (A4)$$

Hence, using (*A*1), (*A*2) and (*A*3),

$$\langle x_i^2\rangle = \int_0^{q_m} (k_B T V_c/2\pi^2 m v_i^2)\, dq$$
$$= (k_B T/2\pi^2\rho)(6\pi^2/V_c)^{1/3}(1/v_i^2),$$

where $\rho$ is the crystal density. Each of the three acoustic branches gives an analogous sum over the Brillouin zone and these are combined together to give (5).

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