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Ultrasonic propagation properties of collagen

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Abstract. Ultrasonic absorption measurements in the range 0·5–56 MHz, and measurements of velocity at 8·87 MHz, were made in collagen suspensions in which molecular integrity was preserved, in a range of concentrations 0·07–0·7% by weight, at 10 and 20 °C. Absorption and velocity values, per unit concentration, exhibited by collagen are much greater in magnitude than those exhibited by globular proteins.

1. Introduction

Collagen is present in most mammalian organs and tissue. It comprises 25–33% of the total tissue protein and, therefore, about 6% of the total body weight, making it the most abundant protein in the body (White *et al* 1968). The local geometrical arrangement and concentration of collagen depends to a large extent upon the function of the tissue. The greatest concentration of collagen is in connective and mechanical supportive tissues, the morphology and function of which have been described in detail (Hall 1965, Bloom and Fawcett 1968, Elden 1968).

The mechanical function of collagen, reflected in its role as a supporting entity, sets this protein apart from the globular proteins. The elastic modulus of connective tissue, for example, is thought to be some 1000 times greater than that of soft tissues (Burton 1967). The density of collagen in its native state, i.e. 1·16–1·33 g cm^{-3} (Hulmes *et al* 1977, Dweltz 1962), is also greater than that of soft tissue, which usually has a density nearer to that of water (Snyder *et al* 1975). Unlike globular proteins, however, collagen exhibits an animal age-dependent variability in its physical and chemical properties (Viidik 1973). It has been suggested (Fields and Dunn 1973) that the unique chemical and mechanical properties of collagen among proteins, and the varied structural characteristics with which it endows tissues, appreciably influence the acoustic properties of tissues. Some evidence already indicates that the presence of collagen in tissue markedly increases ultrasonic attenuation, absorption and velocity in tissue (O'Brien 1976, 1977; Goss *et al* 1979, 1980; Goss and O'Brien 1979). The present study was conducted to determine the nature of ultrasonic absorption and velocity in suspensions of mammalian collagen and to obtain quantitative relationships with regard to concentration, frequency, and temperature.

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2. Experimental method

2.1. Ultrasonic measurements

Two systems were employed for making the ultrasonic measurements in collagen suspensions. The first, a pulse transmission system, was for the measurement of ultrasonic absorption, yielding an accuracy of ±10%, over the frequency range from 9–62 MHz, and for ultrasonic velocity measurements at 8·87 MHz, providing an accuracy of better than 0·5%. This system is an automated version of the technique introduced by Pellam and Galt (1946) and is described in detail elsewhere (Goss 1978, Kessler *et al* 1971). Diffraction effects (Del Grosso 1964, 1965; Seki *et al* 1956) limit the low frequency range of measurement to about 3 MHz and require that the diameter of the sample chamber be large compared to the diameter of the transducer. Under such conditions, the minimum sample volume that can be measured with acceptable accuracy is approximately 500 ml.

A CW resonance method, employed to make the absorption measurements in the 0·5 to 3 MHz frequency range, requires sample volumes one tenth of the size of those required by the pulse transmission method (Eggers and Funck 1973). This method uses an acoustic resonant cavity of the type described by Eggers and Funck (1975), for which the mechanical Q is proportional to the ultrasonic absorption per wavelength of the liquid filling the cavity. The small sample volume required for this system (about 50 ml) is particularly attractive for measurements of biomacromolecular solutions and suspensions for which larger volumes are economically unattainable. Accuracy of the CW resonance method over the 0·5 to 3 MHz frequency range is considered to be ±10%.

2.2. Sample preparation

Calf skin collagen (freeze dried, $M_w = 360\,000$) was obtained from the Devro Corporation, Sommerville, New Jersey. Amino acid analysis of the Devro material using standard hydroxyproline assay procedures for collagen yielded a purity greater than 90% (S Lees 1977, C A Edwards 1978, personal communications). Since collagen is generally insoluble in water, 3% (0·5 M) acetic acid provides a suitable suspending liquid that does not disturb the molecular integrity or conformation of the specimen, and yields a uniform collagen suspension (T Tsuzuki 1975, personal communication). The acetic acid solution (pH = 3) was prepared by adding 45 ml of glacial acetic acid to the appropriate amount of singly deionised and distilled water to make up a volume of 1·5 litres. A weighed amount of collagen (depending upon the specimen concentration desired) was added to the suspending medium and agitated in a Waring Model 16 blender. Staggered operation of the blender was used to avoid heating of the collagen suspension during the mixing process. The specimen was then slowly stirred on a magnetic stirrer for 3–4 days, while maintained at a temperature of 7–8 °C, to produce a uniform suspension. The specimen was allowed to equilibrate thermally in the ultrasonic chamber tank prior to proceeding with the ultrasonic measurements. Upon completion of the ultrasonic measurement procedure, the suspension was removed from the tank and a small volume (25–50 ml) extracted for precise concentration determination by evaporating over air until dry (usually 48 h), placing in a vacuum desiccator for 24 h and weighing on an Ainsworth Type 10 analytical balance (with an accuracy of ±0·1 mg). The precision in determining the solute concentration of the suspensions is considered to be better than 0·3%.

3. Results and discussion

3.1. General

The ultrasonic absorption and velocity in dilute collagen suspensions at pH 3 were measured as a function of frequency and solute concentration at 10 and 20 °C. The specimen concentrations were limited to the approximate range 0·07 to 0·7% by weight due to the extraordinary viscosity of these suspensions, e.g. 280 poise at 1% (T Tsuzuki 1975, personal communication), for which greater concentrations could interfere with the ability to minimise the effects of thermal and concentration gradients.

The excess frequency-free absorption coefficient, $\Delta\alpha/f^2$, defined as

$$\frac{\Delta\alpha}{f^2} = \frac{\text{total suspension absorption coeff.} - \text{solvent absorption coeff.}}{(\text{frequency})^2} \tag{1a}$$

$$= (\alpha_{\text{suspension}} - \alpha_{\text{solvent}})/f^2 \tag{1b}$$

is a convenient quantity for presenting the data. The measured absorption values were corrected for diffraction effects (Del Grosso 1965). In this study, α_{solvent} is the absorption characteristics of the 3% acetic acid used to suspend the collagen. Absorption data is often expressed in this manner because α/f^2 is a constant for pure Stokes' liquids (liquids for which the only mechanisms of absorption are the classical ones of shear viscosity and thermal conductivity). The excess frequency-free absorption coefficient per unit concentration of solute is

$$\alpha_c = \frac{\Delta\alpha}{f^2}\frac{1}{C_s} \tag{2}$$

where C_s is the concentration by weight (grams per 100 cm^3) of solute in the solvent.

The ultrasonic absorption and velocity of the collagen suspending medium was determined using the same experimental systems employed for the collagen suspension measurements. The absorption values obtained were within 10–15% of those obtained by other investigators (Stumpf and Crum 1966, Jackopin and Yeager 1972). Best fit values of the frequency-free absorption coefficient are used in the computation of the excess absorption due to the collagen solute alone (equation 1).

3.2. Concentration dependence

Knowledge of the concentration dependence of the ultrasonic absorption in proteinaceous preparations is necessary for a complete understanding of the absorption process, since absorption has been shown to be influenced by the relation between the macromolecule and its environment (Carstensen 1960). A linear dependence of absorption on concentration is usually attributed to processes involving interaction between the solvent and solute only, and at the very most is limited to low concentrations (Goss and Dunn 1974). A non-linear dependence of the absorption on the concentration has been attributed to intermolecular interaction processes (Kremkau and Carstensen 1972).

The concentration dependence of the excess frequency-free absorption in collagen suspensions at 10 and 20 °C is shown in figures 1 and 2, respectively (tabulated in tables 1 and 2); a nearly linear relationship is seen to prevail over most of the frequency range treated. Fitting the data at each frequency using least squares analysis to a power function of the form $\Delta\alpha/f^2 = \alpha_f C^x$ provides additional detail. Here $\Delta\alpha/f^2$ is the excess

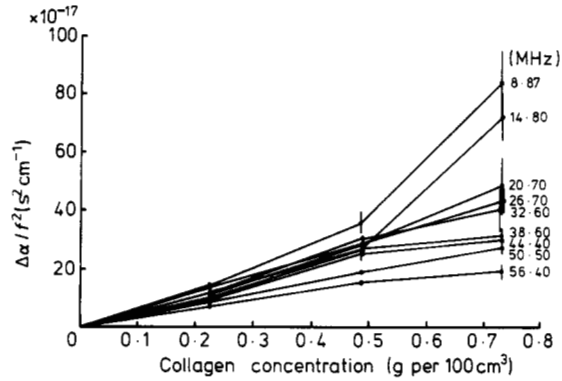

Figure 1. Excess frequency-free absorption coefficient as a function of collagen concentration at 10 °C (bars indicate ± one standard deviation).

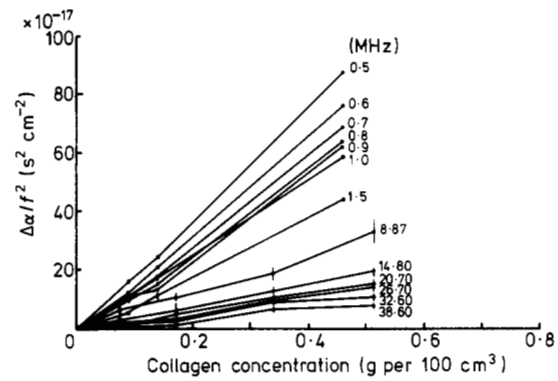

Figure 2. Excess frequency-free absorption coefficient as a function of collagen concentration at 20 °C (bars indicate ± one standard deviation).

Table 1. Dependence of the excess frequency-free absorption coefficient on concentration by weight of collagen at 10 °C (± one standard deviation).

Frequency (MHz)	$\Delta\alpha/f^2 \times 10^{17}$ (s^2 cm^{-1})		
	$C = 0{\cdot}73\%$	$C = 0{\cdot}49\%$	$C = 0{\cdot}23\%$
8·87	83·3 ± 11·2	35·5 ± 3·0	14·0 ± 1·0
14·80	71·9 ± 8·2	25·9 ± 2·0	9·9 ± 1·0
20·70	48·0 ± 9·9	28·2 ± 1·5	11·2 ± 1·0
26·70	43·1 ± 4·3	28·7 ± 1·0	14·0 ± 1·0
32·60	38·9 ± 7·0	30·2 ± 1·0	11·0 ± 2·0
38·60	30·8 ± 2·0	26·6 ± 2·0	10·6 ± 2·0
44·44	30·3 ± 1·0	25·2 ± 1·5	9·9 ± 2·0
50·50	26·8 ± 2·0	18·5 ± 1·0	9·5 ± 1·0
56·44	18·8 ± 2·0	15·4 ± 0·3	7·1 ± 0·4

Table 2. Dependence of the excess frequency-free absorption coefficient on concentration by weight of collagen at 20 °C (± one standard deviation).

Frequency (MHz)	$\Delta\alpha/f^2 \times 10^{17}$ (s^2 cm^{-1})			
	$C = 0{\cdot}46\%$	$C = 0{\cdot}14\%$		$C = 0{\cdot}09\%$
0·5	87·3	24·4		16·0
0·6	75·7	20·4		11·7
0·7	68·6	17·1		10·7
0·8	63·9	14·8		9·8
0·9	62·2	12·9		10·4
1·0	58·8	16·8		10·5
1·5	43·8	11·2		5·5
	$C = 0{\cdot}52\%$	$C = 0{\cdot}34\%$	$C = 0{\cdot}17\%$	$C = 0{\cdot}08\%$
8·87	33·1±4·0	19·3±2·0	10·1±2·0	6·3±2·0
14·80	18·7±1·0	12·5±1·0	5·8±2·0	3·4±1·0
20·70	14·7±1·0	9·3±2·0	4·6±0·9	0·6±0·6
26·70	13·8±2·0	10·0±2·0	3·2±0·3	2·5±1·0
32·60	10·0±2·0	9·2±2·0	2·5±1·0	1·9±0·3
38·60	7·6±1·0	6·6±3·0	1·0±0·2	0·8±0·7
44·44	7·0±0·5	6·9±3·0	0·6±0·7	0·2
50·50	5·3±1·0	5·4±2·0	0·4±0·7	—
56·44	—	3·2±3·0	3·5±0·7	1·3±1·0

frequency-free absorption, α_f is a frequency-dependent constant, C is the collagen concentration in grams per 100 cm^3 and x is the exponent describing the concentration dependence, with $x = 1$ indicating a linear dependence. The results of this fitting are shown in table 3 for all of the measurement frequencies, at both temperatures, together with the cross-correlation coefficient, R, which describes the degree to which the data fit the derived function ($R = \sigma_{xy}^2/\sigma_x\sigma_y$, where σ_x and σ_y are the standard deviations of the x and y arrays, respectively, and σ_{xy} is the covariance), with $R = 1$ signifying a perfect fit to the data. The mean exponents at the two temperatures are nearly identical, i.e. 1·1. It appears that the concentration dependence (for concentration less than 1% by weight) of excess absorption in collagen may be slightly non-linear, and has a small observed temperature dependence.

The results of the present study indicate a non-linear concentration dependence in the concentration range from about 0·07 to 0·7%. Examination of a more concentrated collagen suspension (3·7%) using the transient thermoelectric technique at 0·5 MHz at 37 °C reported elsewhere (Goss 1978) yields an acoustic absorption coefficient of 0·005 cm^{-1}. This data, when fitted to the lower concentration data already presented, may be described by a power dependence exponent of 1·3, and is further evidence that a non-linear dependence of absorption on concentration is present in such suspensions of collagen.

Linear, or nearly linear, behaviour of the absorption coefficient, with varying protein concentration, has previously been observed, predominantly in aqueous solutions of blood proteins over restricted concentration ranges. Carstensen and Schwan (1959) earlier reported that the ultrasonic absorption in haemoglobin solutions is linear to approximately 15 g per 100 cm^3 (15% by weight). This finding was further documented for solutions of uncrystallised and twice-crystallised haemoglobin (O'Brien and

Table 3. Best power fit exponents at 10 °C and 20 °C for concentration dependence of absorption in dilute collagen suspensions.

	10 °C		20 °C	
Frequency	Exponent	Fit, R	Exponent	Fit, R
0·5	—	—	1·05	0·999
0·6	—	—	1·14	0·999
0·7	—	—	1·14	0·999
0·8	—	—	1·16	0·998
0·9	—	—	1·12	0·997
1·0	—	—	1·05	0·999
1·5	—	—	1·25	0·997
8·87	1·5	0·989	0·856	0·986
14·80	1·66	0·982	0·907	0·993
20·70	1·25	0·999	1·60	0·976
26·70	0·971	0·999	0·968	0·960
32·60	1·12	0·986	0·983	0·949
38·60	0·961	0·974	1·35	0·936
44·40	1·00	0·980	—	—
50·50	0·897	0·999	—	—
56·40	0·866	0·987	—	—
	Mean: $C^{1\cdot14\pm0\cdot28}$		Mean: $C^{1\cdot12\pm0\cdot19}$	

Dunn 1972), though the concentration at which the excess absorption coefficient deviated from linearity was found to be dependent on the purity of the sample. A linear dependence of the excess absorption on concentration has been reported for ovalbumin solutions to 15% (O'Brien 1970), and in the synthetic polymer polyethylene glycol to 21% (Kessler *et al* 1970). Thus, the linear concentration dependence of the excess absorption coefficient has been considered to prevail for differing molecular structures, particularly when close inspection over a relatively broad concentration range has not been of paramount importance. However, when the concentration range was extended, a non-linear dependence of the excess absorption was found for bovine serum albumin in aqueous solution. In the concentration range 5 to 40% by weight (Goss and Dunn 1974), a concentration dependence to the power 1·2 was observed at 20 °C, over the frequency range from 3·4 to 15 MHz. Also, the concentration dependence of absorption in solutions of the peptide antibiotic bacitracin (Slutsky *et al* 1977) yields a power dependence of 1·2, with deviation from apparent linearity occurring at concentrations, by weight, as low as 2%. The large disparity in molecular weight between bacitracin ($M_w = 1400$) and bovine serum albumin ($M_w = 68\,000$), as well as differences in molecular structure, raises the question of the role of the concentration of a specific molecular species in the absorption process.

Some understanding of the observed non-linearity in this concentration range for collagen may be obtained by a consideration of the size of the collagen molecule, which is believed to be cylindrical in shape, having a 2900 Å axial length, and a 14 Å diameter (in solution), exhibiting a molecular weight of about 360 000 (Boedtker and Doty 1956). As a first approximation, however, the average dimensions of the collagen molecule can be described by the radius of gyration, which is considered to be about 870 Å (Tanford 1961). The concentration by weight at which collagen molecules would begin to touch can then be determined by that concentration at which the centre to centre intermolecular distance becomes equal to twice the radius of gyration; for

collagen this is 0·011%. For the globular protein haemoglobin, which has been shown to exhibit non-linear absorption dependence on concentration at concentrations greater than about 15% (Carstensen and Schwan 1959, O'Brien and Dunn 1972), the concentration at which molecules touch is 90%, considering haemoglobin as a spherical molecule with a molecular radius of gyration and molecular weight, respectively, of 25 Å and 68 000 (Pilz 1973, Tanford 1961). The collagen concentrations investigated ultrasonically in this study are some 7 to 70 times greater than that considered to be the 'touching concentration' for collagen, and touch at concentrations some four orders of magnitude smaller than the globular proteins previously examined. Thus intermolecular interaction, throughout the concentration range investigated ultrasonically in this study, must be considered likely to contribute to the non-linear behaviour.

The collagen concentration dependence of the acoustic velocity in the collagen suspension is linear for both temperatures (figure 3), with the slope and intercept dependent to some degree upon temperature. In addition to the dependence of the

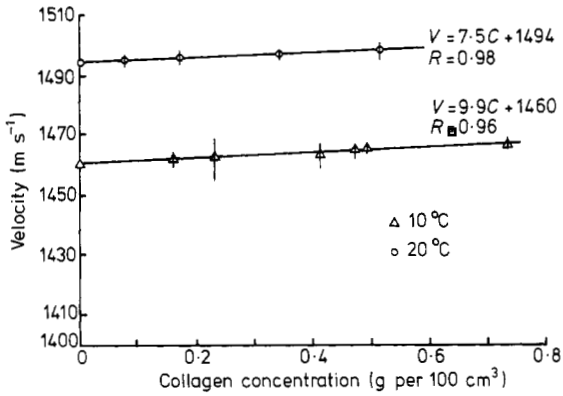

Figure 3. Ultrasonic velocity at 8·87 MHz as a function of collagen concentration at 10 and 20 °C (bars indicate ± one standard deviation).

speed of sound of the suspension upon collagen concentration, there appears to be a slight dependence upon temperature, though confidence in this dependence may be lessened by the limited number of data points measurable over the restricted concentration range. The velocity–concentration slopes, as determined by least squares analysis, for collagen suspensions are, however, at least twice as great as those found in aqueous solutions of globular protein (Goss 1974) and yield a slope of 3·5 $(\mathrm{m\,s^{-1}})/(100\ \mathrm{cm^3\,g^{-1}})$.

Similar analysis of the data for haemoglobin of O'Brien (1970) and Carstensen and Schwan (1959) yield slopes of 3·1 to 3·9 $(\mathrm{m\,s^{-1}})/(100\ \mathrm{cm^3\,g^{-1}})$, respectively, at 10 and 25 °C. Another globular protein, ovalbumin, exhibits a similar dependence on concentration of the velocity (O'Brien 1970). It thus appears that the collagen preparation studied here exhibits a speed of sound per unit concentration in suspension which is at least twice that found for aqueous globular protein solutions.

3.3. Frequency dependence

A useful measure of the frequency dependence in collagen suspensions can be obtained by using the nearly linear concentration dependence of absorption to provide an

average frequency dependence over all concentrations (for which the near linearity prevails) by considering the quantity $\Delta\alpha/Cf^2$ (where C is the collagen concentration in grams per 100 cm^3) as a function of frequency. Figure 4 shows this relationship comprised of the average value (± the standard deviation of the mean results for the different concentrations) of the excess frequency-free absorption per unit collagen concentration as a function of frequency, from 8·87 to 56·4 MHz at 10 °C, and from 0·5 to 56·4 MHz at 20 °C. These yield the frequency dependences of excess frequency-free absorption of $F^{-0.56}$ and $F^{-0.53}$ at 10 and 20 °C, respectively, where F is the frequency in megahertz.

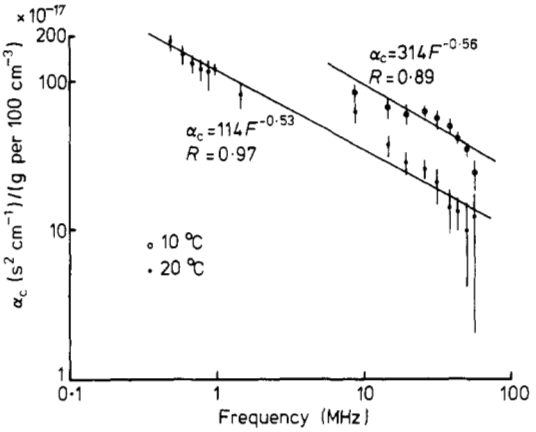

Figure 4. Excess frequency-free absorption coefficient per unit concentration in collagen suspensions as a function of frequency at 10 and 20 °C (bars indicate the standard deviation of the mean results for the different concentrations). F is the frequency in MHz.

The frequency dependence obtained here for collagen in suspension at pH 3 is not unlike that found for other proteins in aqueous solution at neutral pH. Goss and Dunn (1974) determined the frequency dependence of excess frequency-free absorption coefficient in aqueous solutions of bovine serum albumin at neutral pH as $F^{-0.55}$, and similar dependencies have been found for haemoglobin (O'Brien and Dunn 1972, Edmonds *et al* 1970), ovalbumin and DNA (O'Brien 1970) and breast cyst liquid, which may be considered to be an aqueous solution of proteins (mucoproteins and serum albumins) containing various ions (Lang *et al* 1978).

It is interesting to observe that each of these macromolecules possess a very different spatial configuration in solution. Serum albumin and ovalbumin (molecular weights 68 000 and 46 000, respectively), like most globular protein molecules, are constructed of a strongly bonded chain or of tightly folded chains (Foster 1960), forming rigid compact globules at neutral pH. Haemoglobin (molecular weight 68 000) consists of four polypeptide chains which interlink together to form a characteristic globular aggregate. The nucleic acid (molecular weight 10^6–10^9) DNA in aqueous solution exists as a double helix, i.e., two chains held by hydrogen bonding between adjacent bases and wound tightly together, to form a rod-like structure. Collagen, as previously described, takes the form of a triple helix 'cable' of molecular weight 360 000. It appears, then, that structural diversity among biomacromolecules may not appreciably influence the characteristic frequency dependence of ultrasonic absorption. It is interesting to note, too, that the same frequency dependence of absorption found for these molecules has

been determined for dextran, a branched polysaccharide having little in common with the higher order structure of the molecules discussed above (Hawley and Dunn 1969). The common frequency dependence found for all these biopolymers possessing higher order structuring indicates that a common absorption mechanism may be responsible for these absorption manifestations.

The magnitude of the dependence of the ultrasonic absorption on frequency exhibited by collagen in its macromolecular form is of special interest. Figure 5 shows the ultrasonic absorption per wavelength per unit concentration as a function of frequency for collagen and several other biological polymers, i.e. haemoglobin, serum albumin, DNA, and gelatin, the denatured form of collagen. Two curves are shown each for haemoglobin and for serum albumin at two different concentrations, since a non-linear absorption dependence on concentration has been determined for these biopolymers. Although the non-linear dependence of ultrasonic absorption with concentration for collagen suspensions has been demonstrated, for the purposes of figure 5 it may be approximated as linear (to within ±15–20%) for the concentration range dealt with here (less than 1% by weight). Thus, figure 5 suggests that molecular

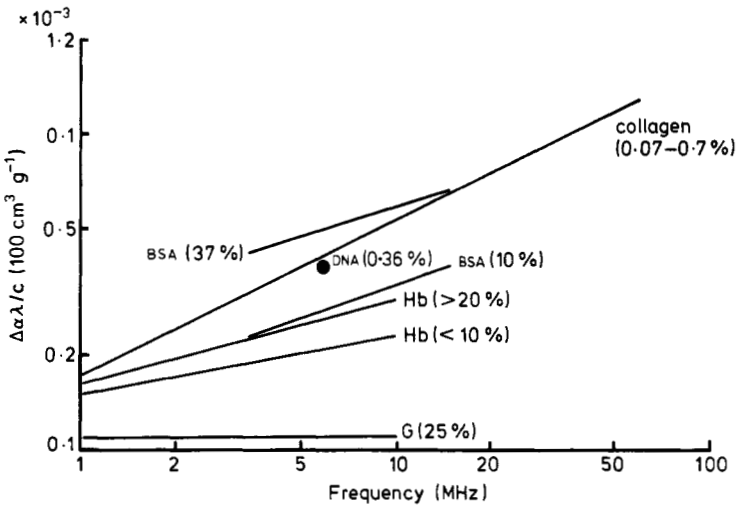

Figure 5. Ultrasonic absorption per wavelength per unit concentration for collagen suspensions and aqueous solutions of other biological polymers. BSA, bovine serum albumin (Goss and Dunn 1974); DNA, deoxyribonucleic acid (O'Brien 1970); Hb., haemoglobin (Cartensen and Schwan 1959); G, gelatin (Pauly and Schwan 1971).

structure, as well as the degree of concentration, has much to do with the ultrasonic absorption characteristics exhibited by a particular biopolymer. For dilute solutions, collagen and DNA are seen to exhibit the greatest absorption per wavelength per unit concentration. For example at 6 MHz, the absorption parameters for collagen and DNA are seen to be more than twice that of a 10% haemoglobin solution, and 45% greater than that of a 10% serum albumin solution. Collagen and DNA also possess the greatest degree of structuring, with collagen consisting of a three-stranded triple helix and DNA the two-stranded double helix. Complete molecular degradation of the helical structure of collagen results in the formation of gelatin, which assumes a random-coil configuration in suspension. Figure 5 shows the results of such degradation of the helical collagen structure, and the attending fragmentation of the respective chains

results in a severe alteration in both magnitude and frequency dependence of the absorption parameters. By increasing the concentration of globular proteins, it is clear that the absorption per unit concentration can be significantly increased to a level greater than that characteristic even of collagen. Thus increasing the concentration by a factor of four (10% to about 40%) increases the absorption per unit concentration by nearly a factor of two at 10 MHz, such that concentrated serum albumin exhibits an absorption per wavelength per unit concentration of the same order of that of dilute collagen suspension.

Acknowledgments

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Résumé

Propriétés de propagation ultrasonique du collagène.

Des mesures d'absorption ultrasonique dans la gamme 0,5–56 MHz, et des mesures de vélocité à 8,87 MHz, furent effectuées dans des suspensions de collagène dans lesquelles l'intégrité moléculaire fut conservée, dans la gamme de concentrations de 0,07 à 0,7% en poids, à 10 et 20 °C. L'absorption et les valeurs de vélocité, par unité de concentration, du collagène sont nettement supérieure en magnitude à celles des protéines globulaires.

Zusammenfassung

Die Ultraschallausbreitungseigenschaften von Kollagen.

Ultraschallabsorptionsmessungen wurden im Bereich von 0,5–56 MHz in Kollagensuspensionen, bei denen die Molekularstruktur des Kollagens unzerstört war, vorgenommen. Die Konzentrationswerte lagen im Bereich von 0,07–0,7 Gw.-% bei 10 und 20 °C. Ebenso wurden Geschwindigkeitsmessungen bei 8,87 MHz durchgeführt. Die Absorptions- und Geschwindigkeitswerte pro Konzentrationseinheit liegen beim Kollagen größenordnungsmäßig wesentlich höher als bei globulären Proteinen.

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