Exp. Eye Res. (1994) 59, 617–627

Relation Between Local Acoustic Parameters and Protein Distribution in Human and Porcine Eye Lenses

C. L. DE KORTE[a*], A. F. W. VAN DER STEEN[a*], J. M. THIJSSEN[a†], J. J. DUINDAM[b], C. OTTO[c] AND G. J. PUPPELS[c]

[a] *Biophysics Laboratory, Institute of Ophthalmology, University Hospital, Nijmegen,* [b] *Netherlands' Ophthalmic Research Institute, Amsterdam and* [c] *Department of Applied Physics, University of Twente, Enschede, The Netherlands*

(*Received Rochester 19 May 1994 and accepted in revised form 1 August 1994*)

The purpose of this study is to characterize the eye lens (human, porcine) by acoustic measurements and to investigate whether relations exist with the local protein content. The acoustic measurements were performed with a 'scanning acoustic microscope' (SAM), operating at a frequency of 20 MHz. At this frequency the lateral resolution in the acoustic images was 150 μm. A double-transmission pulse-echo technique was employed to obtain acoustic parameter images of a central, 1-mm-thick slice of the lenses. The two-dimensional images were derived by ultrasonic spectroscopy displaying the ultrasound velocity, the attenuation at 20 MHz and the slope of the attenuation coefficient between 17 and 23 MHz. The images were summarized by profiles along the optical and equatorial axes of these parameters.

Acoustic parameters were obtained from human lenses ($n = 13$) and porcine lenses ($n = 10$). The protein contents of human lenses were obtained from literature. Additionally, Raman microspectroscopy was used to measure the local protein content of porcine lenses ($n = 3$). Profiles along the optical and equatorial axes were obtained. The relation between the protein content and the acoustic parameters was obtained qualitatively by comparison of the shape of the profiles and, where possible, quantitatively by calculating the Pearson correlation coefficients. Furthermore, the results have been related to a statistical metastudy found in literature in which the relation between collagen, protein and acoustic velocity in various biological tissues was investigated.

The results show a gradual decrease of the magnitude of the acoustic parameters from the centre to the periphery of the porcine eye lens, which is clearly equivalent to the decrease in profiles of the protein content. For the human lenses the acoustic characteristics and the protein content exhibit the same profile, fairly constant in the lens nucleus and decreasing towards the periphery of the lens cortex. Strong positive correlation coefficients for acoustic parameters and protein content for the porcine lens are found.

It is concluded that protein concentration related phenomena can be investigated by measuring the acoustic parameters.

Key words: eye lens; ultrasonic spectroscopy; ultrasound velocity; ultrasound attenuation; Raman microspectroscopy; protein content; correlation.

1. Introduction

Echography, i.e. medical imaging by ultrasound, is a well established diagnostic technique in ophthalmology. The eye lens is a structure which to a certain extent forms an obstruction for the examination of the eye, because of its high reflectivity and absorption. In addition the velocity of ultrasound propagation in the lens is considerably higher than in the surrounding media. A special calibration of the echographic equipment is therefore needed to measure the ocular dimensions with the high accuracy that is needed for calculating the dioptric power of lens implants (cf. Thijssen et al., 1975). In order to be able to achieve an adequate modelling of the eye lens, incorporating also possible inhomogeneities in the acoustic characteristics, the authors have measured the local distribution of ultrasound parameters of the eye lens. The first series of experiments was performed with porcine lenses (van der Steen et al., 1994b). In earlier studies (Thijssen et al., 1983, 1985) it appeared that the relatively strong curvature of the lens surfaces prohibited an accurate measurement of the attenuation. Moreover, the velocity was only measured of the lens as a whole, i.e. no assessment of eventual inhomogeneities was made. The study by van der Steen et al. (1994b) was made with a custom made SAM (scanning acoustic microscope) and a central, 1-mm-thick slice of porcine lenses was measured. Large inhomogeneity of the porcine lenses was observed both for the velocity and the attenuation parameters, which can be summarized as a systematic decrease of the parameters from the centre towards the periphery of the lens. The central profiles (i.e. along optical and equatorial axes) of the acoustic parameter images displayed a clear similarity with those of the protein content in rat lenses and of the refractive index in

* Currently with the Erasmus University Rotterdam and the Interuniversity Cardiology Institute, The Netherlands.

† For correspondence at: Biophysics Laboratory, Institute of Ophthalmology, University Hospital, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

bovine lenses. It was hypothesized that the acoustic and optical parameters of the eye lens are predominantly depending in the local protein content.

In this paper, measurements of the local protein content in porcine lenses are reported. These measurements were performed by means of Raman microspectroscopy (Puppels et al., 1991; De Mul et al., 1993). The Pearson correlation coefficients of the local acoustic parameters and the local protein contents were calculated.

The local acoustic characteristics of the human eye lenses were measured. These results are compared with the data of the protein distribution in human lenses, also measured with Raman microspectrometry, as was derived from an earlier paper (Siebinga et al., 1989).

2. Materials and Methods

Materials

Human eyes ($n = 13$) were donor eyes from donors aged between 19 and 85 years. For measuring the acoustic parameters, the lenses were prepared by cutting the eye at the limbus and reaching the lens from the back. The lens was loosened by cutting the zonula fibres and the lens was taken out. Next the lens was placed in a comb-like cutting device and a 1-mm-thick slice was cut centrally (van der Steen et al., 1994b). Therefore, the lens slice contained the two geometrical axes. By using this method, the cortex of the fresh lenses could not always be prevented from being damaged. The human lens slices were measured 24 to 36 hr post mortem.

Porcine eyes ($n = 10$, age = 4 months) were obtained from the municipal slaughterhouse within 4 hr after death of the animals and prepared in the same way as the human eyes. For measuring the protein concentration, porcine lenses ($n = 3$) were taken out of the eyes using the discussed method, but then fixed for 7 days in a buffered, physiological saline solution with an addition of 4% formalin. This preparation method does not influence the protein content when measured with Raman microspectroscopy (Huizinga et al., 1989). Thereafter, 1-mm-thick slices were cut, containing the two eye axes as before.

Acoustic Microscopy

Instrumentation The acoustic parameters were measured using a SAM (Foster et al., 1984, van der Steen et al., 1994a).

The SAM used in this study (de Korte et al., 1994; van der Steen et al., 1994b) consisted of an ultrasound transducer mounted in a 3-D translation system (stepsize $\Delta X = \Delta Y = 1\ \mu m$, ΔZ continuously adjustable). The lateral resolution of the system is directly related to the transducer used, and was calculated to be 150 μm (transducer V317, Panametrics, central frequency 20 MHz, -6 dB bandwidth 11 MHz, diameter 6 mm, focal length 12·5 mm). The transducer was excited by a short electrical pulse (5 nsec half-power), from a commercial pulser (AVG-3-C, Avtech). The transducer was directed perpendicularly above a flat Plexiglas plate and immersed in a degassed physiological saline solution filling a small water tank. The transmitted ultrasound pulse travelled from the transducer through the tissue, and after reflection off the bottom plate and a second transmission through the lens slice, the echo was received by the transducer. Next the echoes were preamplified by a custom-made amplifier (20 dB, -6 dB bandwidth 0·5–150 MHz) and digitized in 8-bit by a digital oscilloscope (DSA 601, Tectronix) at a 100-MHz sampling rate after passing an antialiasing low pass filter (seventh order Bessel, cut off frequency 70 MHz). An IBM compatible PC controlled the XY-translation of the cross-table and was also interfaced to the oscilloscope. The data were transferred to the PC (optical disk) and, after completion of the measurements, from the PC to a Microvax 3200 (Digital Equipment) for off-line processing.

Data acquisition For measuring the local acoustic parameters of the lens, the slice was placed on the Plexiglas plate and covered by a polyethylene membrane (thickness 3 μm, attenuation $< 0{\cdot}2$ dB in the frequency range used). Next a computer controlled 2-D scan was made (human lens 120×80 points $\delta x = \delta y = 100\ \mu m$, porcine lens 100×80 points $\delta x = \delta y = 150\ \mu m$) with the focal zone of the transducer at the top surface of the Plexiglas. After the scan, the slice was removed and a reference scan was performed at exactly the same positions as the first scan. One scan lasted for 18 min.

Data processing For each point, the three acoustic parameters were calculated (van der Steen et al., 1991, 1994a): the velocity of ultrasound and the attenuation spectrum, which was characterized by the value at 20 MHz (central frequency of the transducer) and the slope of the spectrum between 17 and 23 MHz (i.e. the -6 dB bandwidth of the attenuation spectrum after attenuation cf. van der Steen et al., 1994b).

The three acoustic parameters were displayed in 2-D grey-level images. Next the images were smoothed by applying a 2-D nine-points moving-average filter. The local acoustic parameters were characterized by two profiles, along the optical and equatorial axes of the lens. The profiles were taken horizontally and vertically through the centre of the images (which was determined as the point where the maximum value in the velocity occurred). The equatorial half-profiles were calculated by superimposing the two halves to one axis from centre to equator. Next the data from all lenses were averaged for each position and finally the average profile and $\pm\sigma$ values were calculated. Profiles along the optical axis of the acoustic para-

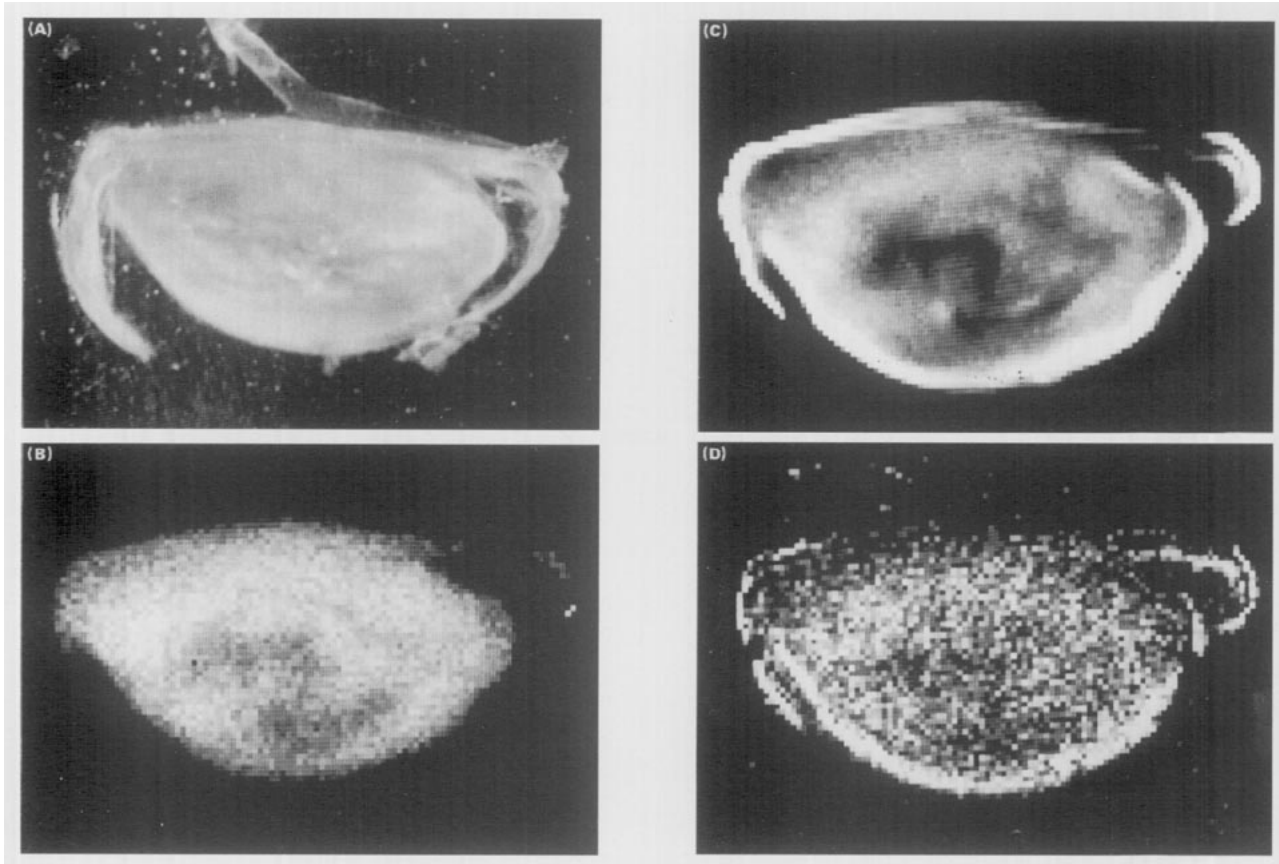

FIG. 1. (A) Optical reflection image of 1-mm-thick, central slice of human lens; (B) corresponding grey scale encoded image of local velocity of ultrasound (range 1500–1700 m sec^{-1}); (C) image of attenuation coefficient at 20 MHz (range 0–30 dB cm^{-1}); (D) image of slope of attenuation coefficient (range 0·0–2·0 dB cm^{-1} MHz^{-1}).

meters of all the lenses were determined and the average profile and the $\pm\sigma$ values were calculated.

Raman microspectroscopy

Instrumentation The local protein content was measured using Raman microspectroscopy. Raman spectra were recorded with a confocal Raman microspectrometer (CRM, cf. Puppels et al., 1991). The excitation wavelength was 660 nm. The light source was a tunable dye laser (model 375 B, Spectra Physics), operated with the laser dye 4-dicyanomethylene-2-methyl-6-[*p*-dimethylamino)styryl]-4-*H*-pyran (Palys et al., 1992), which was pumped by an argon laser (Model 52, Coherent). A water immersion objective (Plan Neofluar, 63×, NA = 1·2, Zeiss) was employed to focus the laser beam in the lens slices and to collect the light scattered by the lens.

Data acquisition Raman spectra were measured at a series of positions along the full optical ($\sim$ 20 points) and equatorial ($\sim$ 30 points) axes of the porcine eye lens ($\delta x = \delta y = 300\ \mu m$). Constant depth within the slice was maintained by the following method: first the system was focused at the top surface of the slice and then the distance between surface and objective was decreased by 50 μm, i.e. the focus was located at a fixed depth within the lens. Each Raman spectrum was obtained in 40 sec. A complete profile took approximately 60 min.

Data processing The Raman spectra were corrected for the wavelength dependency of the light transmission of the CRM. Furthermore, inhomogeneity of the pixel sensitivity of the camera (Wright Instruments, U.K., model AT1 fitted with a P8603 EEV CCD chip) used in the CRM was compensated for (Puppels et al., 1991). The estimation of the Raman intensities at 2935 cm^{-1} and 3390 cm^{-1}, after background subtraction, was made by using custom made analysis software (RAMPAC, cf. De Mul et al., 1993). The absolute water content was calculated from the Raman intensities by using the method described by Huizinga et al. (1989). The protein content was, finally, obtained by simply taking the complement of the water concentration, thereby neglecting the presence of lipids in the lens ($< 2\%$). The profiles of the protein concentration were normalized by rescaling along the position axis. The transition between the lens and water was taken at both sides at a concentration of 0·20 g cm^{-3}. For all lenses the distance between the edges was made identical. The data of both halves of the equatorial profile of all three eyes were superimposed to one axis from centre to equator. For both the profiles along the equatorial and optical axis the measured protein contents were

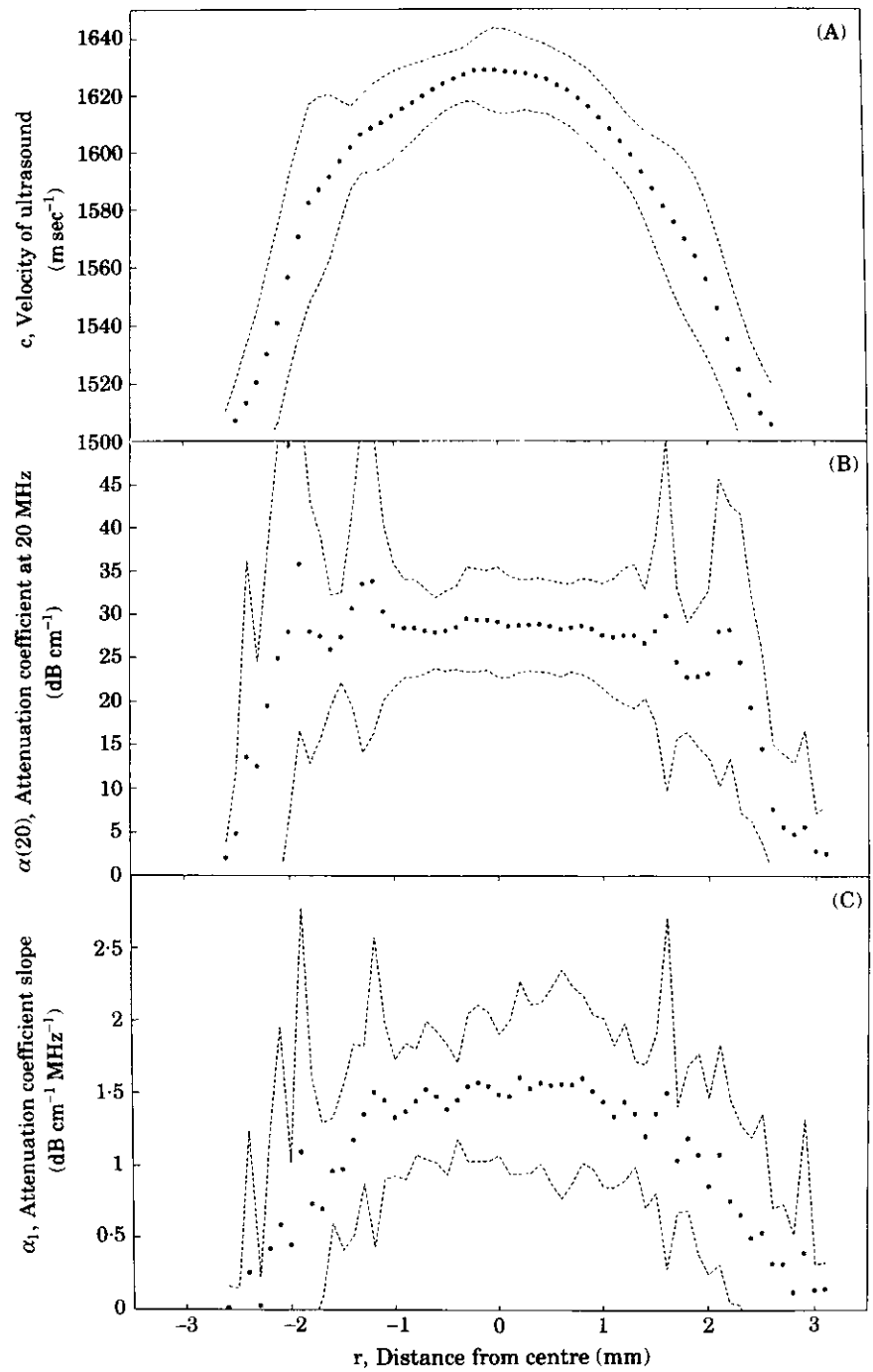

FIG. 2. Profiles of acoustic parameters along optical axis in human lenses ($n = 13$), solid points; mean values, dotted lines; ±S.D.: (A) velocity of ultrasound; (B) attenuation coefficient at 20 MHz; (C) attenuation coefficient slope.

conglomerated within successive intervals of 450 μm, and the mean value and $\pm\sigma$ of the protein content within each interval were calculated. In this way a similar interspacing along the position axis for the protein content and acoustic parameters occurred. The mean value of the protein profile along the optical axis was calculated and is in accordance with the bulk values published in the literature.

Relational statistics The profiles of the acoustic parameters and the local protein contents along the optical and equatorial axes are displayed in graphs, so a qualitative comparison can be made. A quantitative comparison was performed in two ways.

In the first procedure, the Pearson correlation coefficients were calculated to find a quantitative relation between the protein contents and the acoustic parameters in porcine lenses. The Pearson correlation coefficient between the acoustic parameter and protein content profiles of porcine lenses was based on the data points of the profile along the optical axis. Only

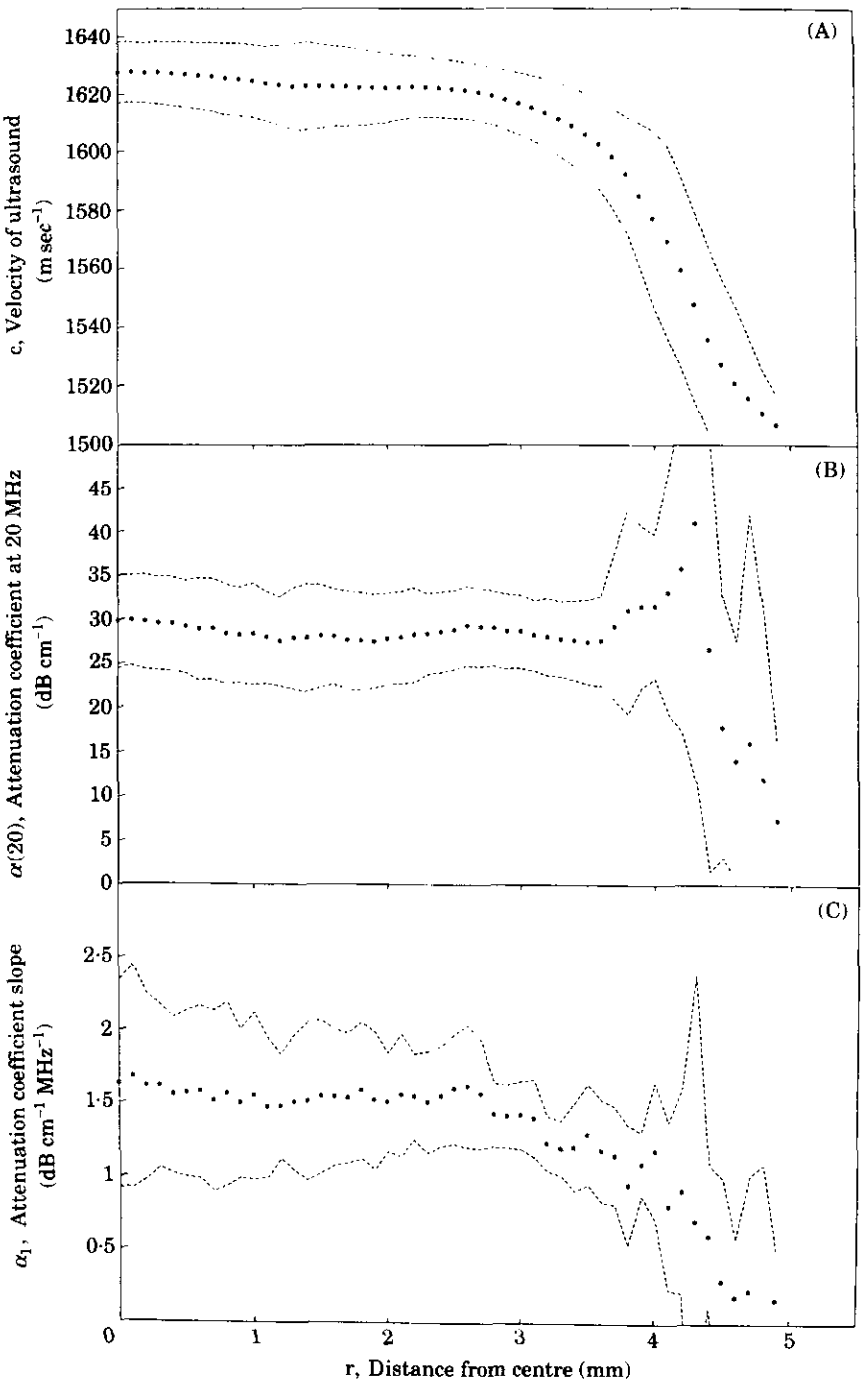

FIG. 3. Half-profiles of acoustic parameters along equatorial axis in human lenses ($n = 13$); same parameters as in Fig. 2.

lenses with an intact posterior and anterior cortex were taken ($n = 5$). The acoustic parameter profiles were transformed in profiles with a 450 μm interspacing along the position axis by averaging three points. In this way the Pearson correlation coefficient could be calculated directly. The data of the local acoustic parameters and the local protein content of human lenses (Siebinga et al., 1991) are not at all normally distributed, so calculation of the Pearson correlation coefficient would be incorrect.

In the second approach the velocity profile was calculated from the protein content profile. As was discussed in an earlier paper (van der Steen et al., 1994b) a relation between the velocity of sound and the protein content of biological tissues was extrapolated by Goss et al. (1980). In the latter study data of several biological tissues and acquired at varying temperatures (average 27°C), were taken from literature and the following formula could be fitted to the data

$$c = c_0 + 3{\cdot}2 \times 10^2 P + 6{\cdot}5 \times 10^2 C \text{ (m sec}^{-1}\text{)} \quad (1)$$

where c = speed of sound, c_0 = speed of sound in physiological saline solution, P = concentration globu-

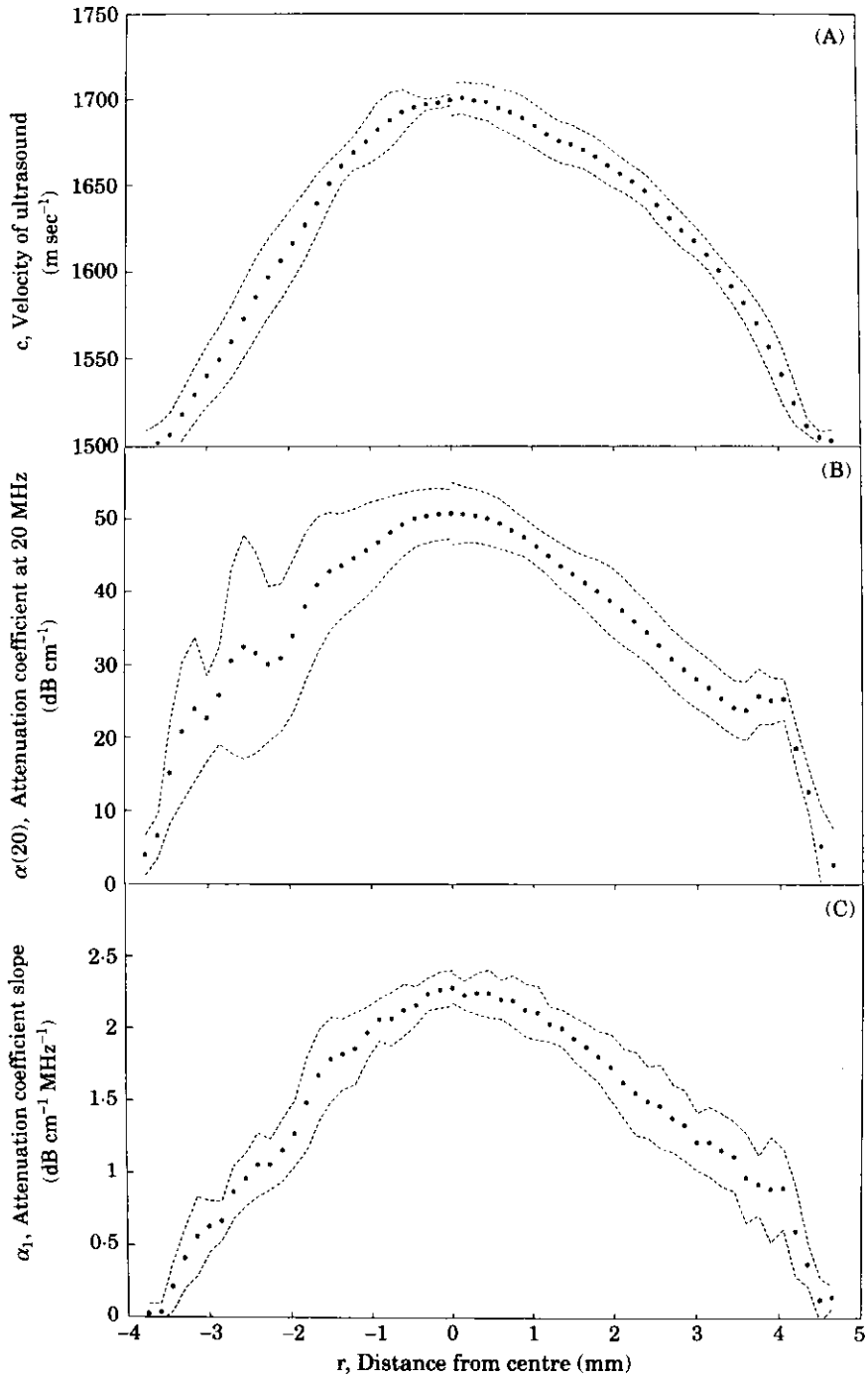

FIG. 4. Profiles of acoustic parameters along optical axis in porcine lenses ($n = 10$); same parameters as Fig. 2.

lar protein (g cm^{-3}) and C = concentration collagen (g cm^{-3}).

Owing to the absence of collagen in the lens (Goss et al., 1980), the protein concentration is fully determined by globular proteins so eqn (1) reduces to (c_0 is taken to be 1500 m sec^{-1} at 20°C; van der Steen et al., 1994b)

$$c = 1500 + 3{\cdot}2 \times 10^2 P \text{ (m sec}^{-1}\text{)}. \quad (2)$$

For human lenses the protein profile measured by Siebinga et al. (1991) was taken, after rescaling of the position axis (because of a difference in lens size).

3. Results

A photograph of a slice of the human lens is shown in Fig. 1(A). The corresponding 2-D acoustic images in Fig. 1(B) to (D) show the velocity of ultrasound (grey levels scaled from black to white over the range 1500 to 1650 m sec^{-1}), the attenuation coefficient at

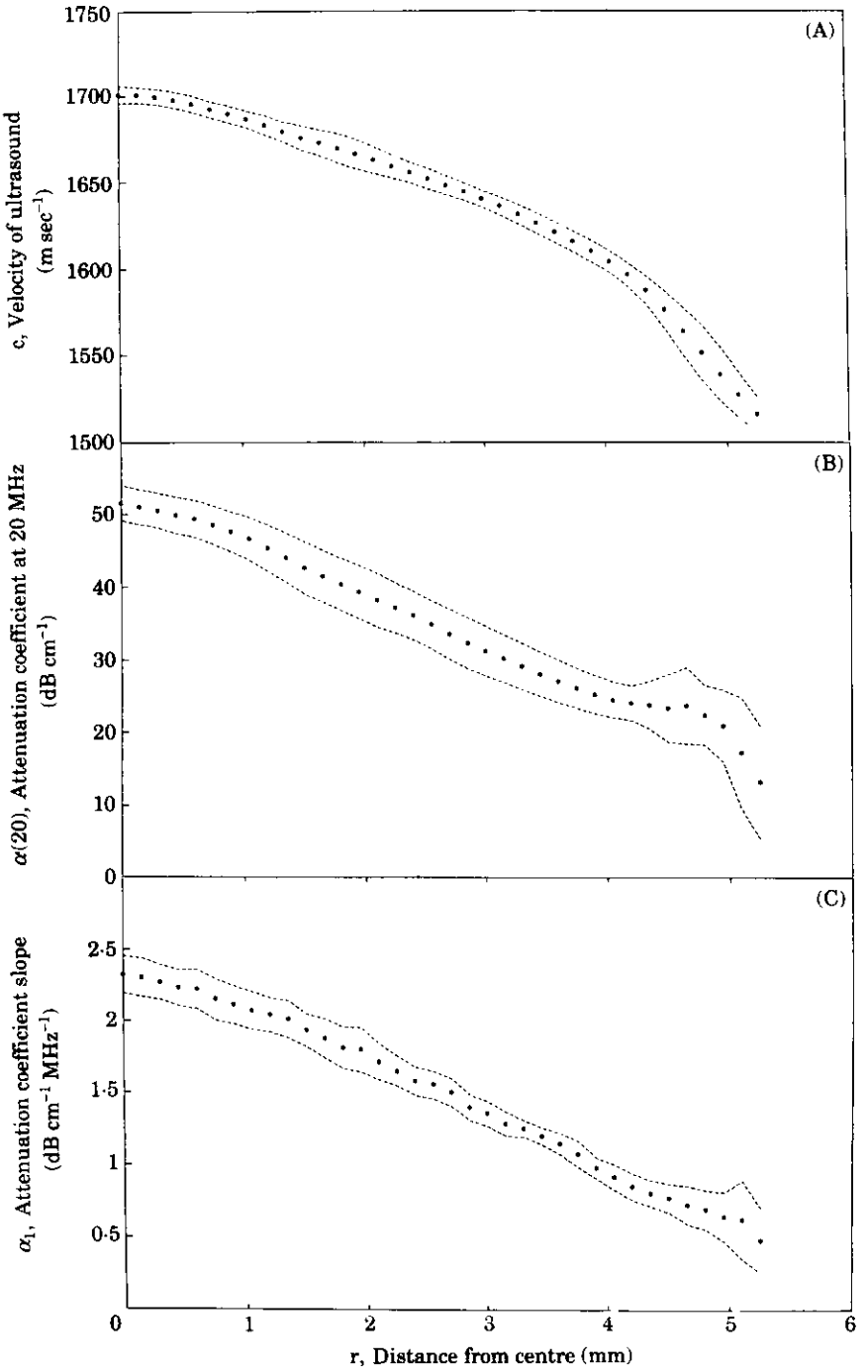

FIG. 5. Half-profiles of acoustic parameters along equatorial axis in porcine lenses ($n = 10$); same parameters as Fig. 2.

20 MHz (range 0 to 30 dB cm^{-1}) and the attenuation coefficient slope [range 0·0–2·0 dB cm^{-1} MHz^{-1}). Apart from some irregularities [also present in the optical slice, Fig. 1(A)] the nucleus does not display a large gradient in the acoustic parameters. This observation is confirmed by the plots of the profiles along the optical axis (Fig. 2) and the equatorial half-profiles (Fig. 3) of the same parameters. In these figures the mean of the depicted parameters of all lenses ($n = 13$) and the lines of plus/minus one standard deviation are plotted (dashed lines). The irregularities at ± 2 mm and at 4 mm distance from the nuclear centre in Figs 2 and 3, respectively, are located at either the transition from cortex to nucleus, or from the cortex to the bathing fluid. The cortex could not be prevented from loosening from the nucleus in some cases.

The acoustic parameters of the porcine lenses are summarized in Figs 4 and 5. The profiles of the velocity, the attenuation coefficient at 20 MHz and the attenuation coefficient slope along the optical axis, are shown in Fig. 4(A) to (C), respectively. The equatorial half-profile of these parameters is displayed in Fig.

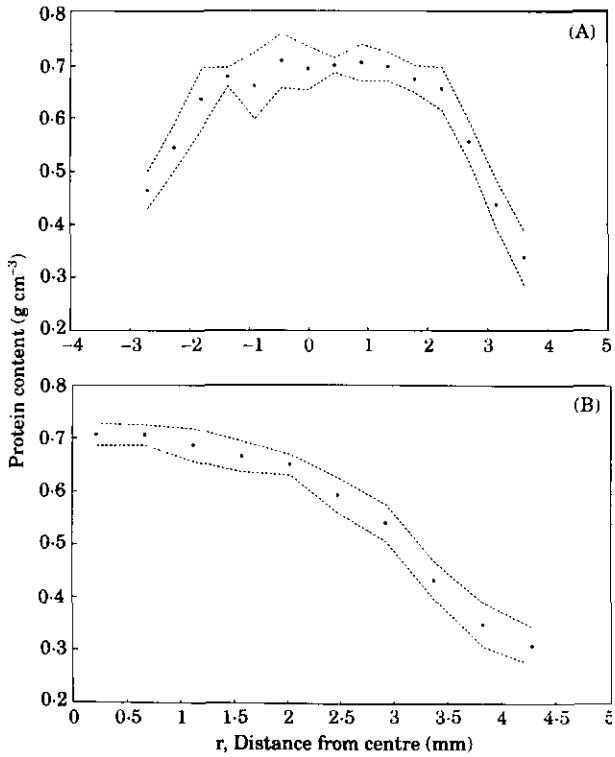

FIG. 6. Profiles of protein content in porcine lenses ($n = 3$) determined with Raman microspectroscopy: (A) optical axis profile; (B) equatorial half-profile.

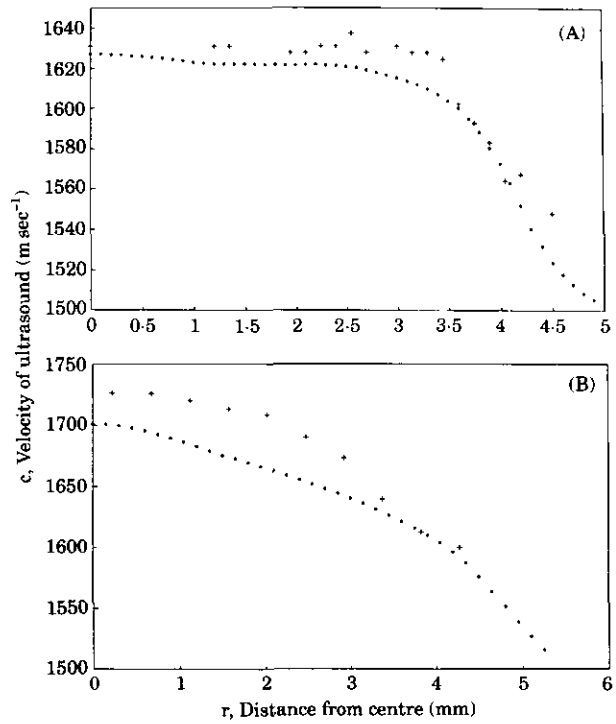

FIG. 8. (A) Equatorial half-profile of velocity of ultrasound, measured (*) and calculated (+) in the human lens; (B) equatorial half-profile of velocity of ultrasound, measured (*) and calculated (+) in the porcine lens.

5(A) to (C). (These data were shown with a second order curve fit in van der Steen et al., 1994b.)

The measured local protein content in the porcine lens is shown in Fig. 6(A) and (B) in profiles, along optical and equatorial axes, respectively. The profiles of the protein distribution have a shape and appearance similar to the acoustic parameter profiles in Figs 4 and 5. The mean value along the optical axis profile is 0·612 g cm^{-3} (i.e. approximately 60%). This value compares well with known values from literature.

The human acoustic parameter profiles in Figs 2 and 3 are compared with protein content data (Fig. 7) taken from the literature (Siebinga et al., 1991). The data in that paper were obtained from an 8-year-old subject. The protein content was calculated from these data as 100% minus the water content. The

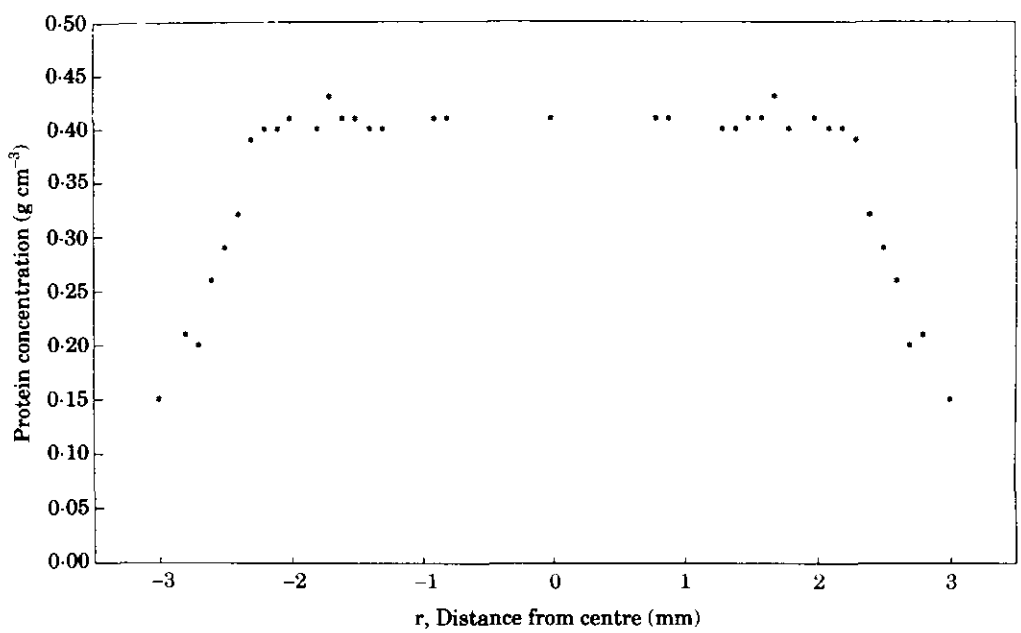

FIG. 7. Protein concentration (equatorial profile) in 8-year-old human lens (from Siebinga et al., 1991).

TABLE I

Pearson correlation coefficients for porcine lenses ($n = 5$)

	c	$\alpha(20)$	α_1	P
Sound velocity (c)	1·00	0·97	0·92	0·89
Attenuation coefficient at 20 MHz ($\alpha(20)$)	—	1·00	0·91	0·83
Attenuation coefficient slope (α_1)	—	—	1·00	0·80
Protein content (P) ($n = 3$)	—	—	—	1·00

calculated equatorial velocity half-profile out of the protein content profile is displayed in Fig. 8(A) together with the velocity data from Fig. 3. For the porcine lens, the equatorial velocity half-profile as calculated out of the measured local protein content is displayed with the measured equatorial velocity profile in Fig. 8(B).

The Pearson correlation coefficients, calculated for the porcine lens, are listed in Table I. A very high and significant ($P < 0{\cdot}01$) correlation is not only found between the acoustic parameters (> 92%), but also between these parameters and the protein contents (> 80%).

4. Discussion

Three acoustic parameters were measured in human and porcine eye lenses with a high resolution (respectively 100 μm and 150 μm). The results show that the variation of the acoustic parameters in the human eye lens is less than in the porcine lens (van der Steen et al., 1994b). As can be seen, the optical axis profiles of the two species look similar (Figs 2 and 4), but the equatorial profiles are different in shape (Figs 3 and 5). In the porcine lens the acoustic parameters decrease from centre to equator, contrary to the human lens where the parameters have approximately the same value over a wide range (nucleus diameter).

The variability in the human data is much greater than in the porcine data. This might be caused by the variations in age of the lenses. The human lenses were in a range of 19 to 85 years unlike the porcine lenses, all approximately 4 months old. The high variance in the acoustic properties is in correspondence with the variation in the protein content in human lenses (Huizinga et al., 1989).

The local protein content in the porcine lens was measured (Fig. 6). The values obtained show a varying protein content along the optical and equatorial axis. Only one paper was found describing a value for the protein content in the cortex and in the nucleus. Deussen et al. (1989) estimated the water content in the cortex to be 0·80 g cm^{-3} and in the nucleus 0·60 g cm^{-3} (i.e. 20 and 40% protein content respectively). These values are the average values for the total nucleus volume and the total cortex volume and will therefore be lower than the average value of the profile due to the geometrical properties. In this way a direct comparison cannot be made.

The quantitative comparison of the protein content and the acoustic parameters of the porcine lenses by calculating the Pearson correlation coefficients shows that the four parameters are strongly and positively correlated (Table I). A strong positive correlation between the two attenuation parameters was also found in other tissues (Oosterveld et al., 1991; Romijn et al., 1991; Thijssen et al., 1993; van der Steen et al., 1994a). The correlation between the ultrasound velocity and the attenuation parameters has not yet been described in the literature. Recently van der Steen et al. (1994c) found that there is no correlation between local velocity of ultrasound and the local attenuation parameters in liver. The strong correlation between the attenuation parameters and protein content has not yet been described in literature. In a statistical metastudy Goss et al. (1980) found a systematic dependence of the velocity of ultrasound on the protein content.

The velocity of ultrasound in the human lens, calculated from the known protein content (Siebinga et al., 1991) by using Eqn (1), was rescaled along the position axis because of the difference in lens size. Fig. 8(A) and (B) show that the shapes of the calculated profiles, both human and porcine, are closely related to the measured profile. The absolute values of the calculated profiles are higher than the value of the measured profiles. This discrepancy can be caused by a difference in temperature. The proportionality factor in the Goss formula eqn (1) was derived for tissues at 27°C and the velocity of ultrasound was determined at 20°C. The velocity of ultrasound will increase linearly with increasing temperature (Wells, 1969). Values found in literature alter from 1·0 m sec^{-1} K^{-1} in the lens (Rivara and Sanna, 1962) to 3·0 and 5·0 m sec^{-1} K^{-1} in water (Hueter and Bolt, 1955; Kinsler and Fry, 1962; respectively). The difference in temperature is 7°C and this will cause a difference of 10 to 35 m sec^{-1} in velocity. The observed difference between the calculated and measured velocity profile is in the same order. For an exact determination of the relation between the protein content and the velocity of ultrasound in vivo the measurements should be performed at 37°C.

When the temperature effect is taken into account, it can be concluded that the local velocity of sound and local protein content in the human and porcine lens can be calculated when one of these two parameters is known. So, not only the acoustic parameters are strongly related to each other, but also the acoustic parameters to the protein content. This means that all the profiles can be predicted from only one profile.

Changes in the protein content and distribution appear to be associated with cataract. Siebinga et al. (1991) and Deussen and Pau (1989) concluded that

the nuclear protein content is decreasing with increasing age. Another protein related phenomenon is 'cold cataract'. This reversible cataract is due to a phase separation of the protein–solvent mixture of the cytoplasm (Tanaka and Benedek, 1975; Tanaka et al., 1977; Clark and Benedek, 1980; Delaye et al., 1981). Cataract is also associated with mutations in certain lens proteins (Zigman et al. 1991; Mota et al., 1992; Rao et al., 1992). These phenomena could be investigated by measuring one of the acoustic parameters. The ultrasound velocity cannot be determined directly in vivo, but the attenuation coefficient slope per unit of time, which is a combination of the velocity and attenuation, as described by Goedegebure et al. (1992), could be determined. The latter parameter can be determined by measuring the backscattering out of the lens or the reflections of the anterior and posterior interface. The more so because there is a positive correlation between the velocity and attenuation the attenuation coefficient slope per unit time might be a useful parameter for detecting protein content changes due to ageing of the lens.

Acknowledgements

This work has been supported by a grant from the Dutch Cancer Fund—Koningin Wilhelmina Fonds (NUKC 89-03). The human eye tissues were obtained from Eurotransplant and the Cornea Transplantation Department of The Netherlands Ophthalmic Research Institute. These institutions are acknowledged for supplying donor material.

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