Ultrasound in Med. & Biol., Vol. 5, pp. 159–165
Pergamon Press Ltd., 1979. Printed in Great Britain

ULTRASONIC PROPAGATION THROUGH FIXED AND UNFIXED TISSUES

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(*First received* 9 *November* 1978; *and in final form* 14 *March* 1979)

Abstract—Ultrasonic attenuation and backscattering coefficients and speed of sound were determined experimentally, as functions of frequency, in samples of fresh mammalian brain, liver and spleen, and in the same specimens fixed histochemically. It is observed that 4% formalin and 5% potassium dichromate are greatly superior to ethyl alcohol for consistently preserving the ultrasonic propagation properties to within only a few percent of those of the original fresh, unfixed material.

Key words: Absorption, Acoustic, Attenuation, Fixatives, Scattering, Tissue preservation, Ultrasonics, Velocity.

INTRODUCTION

As ultrasound becomes increasingly employed in medical diagnosis and therapy its effectiveness and safe employment become more pertinent issues for discussion. Such discussions often lead to the conclusion that further progress is hampered by the gross lack of basic data on crucial propagation characteristics such as absorption, attenuation and scattering coefficients, velocity and impedance, as functions of the basic variables such as temperature and frequency (Goss *et al.*, 1978a, b). Major problems in the accumulation of the necessary information have been those of obtaining tissue specimens of particular interest, of handling them in ways that facilitate measurement yet yield results characteristic of *in vivo* tissues, and of being able to confirm these data at a later time with perhaps other instrumentation, other methods and other specimens. A partial solution to these problems involves preservation of morphology of tissue specimens by fixation methods. Samples so treated may be measured repeatedly over very long periods of time, and even transported over appreciable distances to other laboratory facilities. Standard uniform preparations commercially available for local reference purposes may also be possible.

In this context the value of histochemical fixation is limited by the degree and consistency with which it affects the ultrasonic propagation properties of the treated tissues. There appear to have been few published reports of work specifically relating to this problem. The attenuation studies of fixed dog lung by Bauld and Schwan (1974) can be compared to those by Dunn (1974) on freshly excised specimens to show that absolute values may not be greatly altered, but that the spread of values of the former is much greater than that of the latter. However, as lung is a particularly difficult organ to deal with acoustically and, as different measuring methods were employed in these two studies, little else can be concluded. Tanaka (1969) and coworkers have provided evidence that ultrasonic attenuation and impedance of both normal and abnormal brain tissue are only slightly changed by formalin fixation, the latter resulting from corresponding increasing density and decreasing speed of sound. Formalin fixation is reported to decrease the speed of sound in human brain by 0.4% (Kremkau *et al.*, 1976).

The data of Chivers and Hill (1974) for the attenuation of selected formalin fixed human tissues, when compared with other data in the literature, show tendencies towards the upper limits of the range of values found for fresh tissues. Calderon *et al.* (1976) comment that they observed no change in the attenuation of ultrasound by breast tissue for fixation periods of up to one week but that an increase in the acoustic contrast occurred after two months in 10% formalin.

With regard to the effects of fixation on the scattering properties of tissues, Shung and Reid (1977) claim that formalin fixation increases the backscattering coefficient of bovine liver by more than 400% at 3 MHz.

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This result, which seems to be in conflict with the observations of Lele and Senapati (1977), is also somewhat surprising when the work of Tanaka *et al.* (1969) is considered, and may raise interesting questions regarding the specific aspects of the tissue structure that have been affected.

The present study was undertaken with the view towards more clearly establishing the degree to which histochemical fixation changes the ultrasonic propagation properties of a variety of tissues, and comparing several fixation methods. It is possible to identify three main categories of histochemical fixatives (Pearse, 1968; Thompson, 1969), roughly defined according to their action on the protein components of tissues, viz. protein cross-linking agents, protein denaturing agents, and protein precipitating agents. For this investigation it was convenient to limit the number of fixatives by choosing one from each of the three categories. Simple fixatives (as opposed to mixtures of compounds) were chosen in order to exhibit extremes, so that their different effects (if any) on the ultrasonic propagation properties could be more easily demonstrated. Thus 4% formalin, absolute ethyl alcohol, and 5% potassium dichromate were employed. Formalin has little effect upon neutral fats, but preserves proteins by attaching itself to side groups of certain amino acids, thereby denaturing protein and rendering it insoluble (Thompson, 1966). Although the reactions are complex, the principal action of formalin may be upon amino groups of lysine and glutamine, with the binding usually occurring between such amino groups in adjacent protein chains. Alcohol is a strong reducing agent. It denatures protein by coagulation and rapid dehydration (an action similar to the effect of heat upon tissues). During this process glycogen is preserved, as are sulfhydryl groups of proteins, though most lipids are dissolved. Potassium dichromate is a strong oxidizing agent, precipitating protein (possibly by effects upon tyrosine, tryptophan and histidine) while preserving carbohydrates, glycogen and lipids.

METHODS

1. *Ultrasonic*

The method employed for the attenuation measurements has been described previously (Papadakis *et al.*, 1973; Chivers and Hill, 1975; Bamber *et al.*, 1977). Briefly, a pulse of ultrasound, emitted by an appropriate transducer, is reflected from a plane surface situated normal to the direction of sound propagation. The reflected pulse, which is isolated by a time-gating circuit, is received by the same transducer, amplified, and fed to a spectrum analyser which displays its Fourier transform. The attenuation due to a double traverse of the tissue specimen, cut to have plane parallel surfaces, is the difference between the received echo signal with and without the sample interposed between transducer and plane reflector, provided that a logarithmic spectral display of amplitude is employed. One plane transducer, having a nominal frequency of 10 MHz, was used to cover the frequency range 1–7 MHz. A gate duration of 20 μs was chosen such that its spectrum would not affect that of the echo pulse, when used with an intermediate frequency bandwidth of 100 kHz.

The 7 mm diameter transducer was positioned 45 mm from the centre of the specimen, where it had a 6dB "echo" beam width that varied from 3.5 mm (1 MHz) to 2 mm (7 MHz). The reference plane surface was placed 70 mm from the transducer and the corresponding diffraction corrections were less than 0.05 dB cm^{-1} ($\simeq$1%), i.e. a negligible amount. The methodology was tested and verified by making measurements with materials of known acoustic properties, viz. castor oil and cotton seed oil (Dunn and Breyer, 1962). This procedure also indicated that the precision in the total attenuation obtained by this method was $\pm$0.3 dB ($\simeq$3% for the thinnest specimens). A detailed discussion of the errors associated with this method may be found elsewhere (Bamber 1979), but the accuracy is generally limited (for acoustically homogeneous media) by the inability to specify the acoustic path length in the specimen to better than about $\pm$10%. As discussed by Miller *et al.* (1976), real tissues are not acoustically homogeneous and therefore give rise to additional errors due to phase cancellation at the receiver. Attempts to minimise such errors will be referred to later in this paper.

A small computer, linked to the system, facilitated data collection and analysis.

The same experimental system was used to make measurements of the speed of sound in the tissue specimens. By observing the shift in the position of the echo from the plane

reflector, due to interposing the specimen, the average velocity over the tissue path length may be calculated relative to the speed of sound in the surrounding medium (usually water). The accuracy of these measurements is again limited primarily by the uncertainty as to the acoustical path length in the specimen, and is estimated as less than ±4% for specimens measured in alcohol and less than ±1% for all other specimens.

The method used in making the backscattering observations has also been described previously (Nicholas and Hill, 1975; Bamber *et al.*, 1977). Briefly, an approximately circular cylindrical tissue specimen was positioned with its long axis normal to and directly in the path of the sound beam, the acoustic coupling medium being water. The transducer was employed as both the transmitter and the receiver. The specimen was rotated about its cylindrical axis such that the amplitude of the backscattered signal could be obtained from all angles. A time gate, centered on the axis of rotation, selected a portion of the echo signal, the total such received signal thus corresponding to the backscattering from a volume of tissue determined by the beam width and the time gate, which in this instance was 5 μs. The signal is processed to provide the amplitude of the volume-backscattered signal, at a particular ultrasonic frequency, as a function of angular rotation. Scattering patterns so obtained appear to be characteristic of the tissue viewed (Nicholas and Hill, 1975).

For this investigation the backscattering cross section per unit volume was computed from the following equation and then averaged, for a full revolution of the sample, over 1000 evenly spaced tissue orientations,

$$\eta_{bs} = \frac{W_s}{W_R} \cdot \frac{4\alpha R}{\Omega\, e^{-4r\alpha}(e^{c\tau\alpha} - e^{-c\tau\alpha})} \qquad (1)$$

where W_s and W_R are the measured power scattered from the tissue and the total power returned by a plane reflector (of reflection coefficient R), after conversion from a logarithmic to a linear scale, respectively, α is the amplitude attenuation coefficient of the tissue, r is the radius of the tissue cylinder, c is the velocity of sound in the tissue, τ is the duration of the time gate and Ω is the solid angle subtended by the transducer face at the centre of the specimen. The derivation of equation 1, including a detailed discussion of assumptions, is given by Bamber (1979) and is similar to that given by Sigelmann and Reid (1973) in describing their substitution method of measuring scattering from blood.

Three plane transducers were used to make backscattering measurements at their nominal frequencies, viz. 1 MHz, 2.5 MHz, and 4 MHz. For each transducer the distance of the transducer face to the centre of the specimen was chosen to be a few millimeters into the Fraunhofer diffraction zone. The 22 mm diameter 1 MHz transducer had a 6 dB beam width of 6 mm at, and was positioned 50 mm from, the centre of the specimen. For the 2.5 MHz transducer these values are, respectively, 15 mm diameter, 4.5 mm beam width, 76 mm distance; and for the 4 MHz transducer they are 15 mm, 3 mm and 126 mm.

Errors in the scattering measurements are due predominantly to uncertainties as to the length of the scattering volume ($\simeq \pm 12\%$) and as to the value of the attenuation coefficient (an error of 1 dB cm^{-1} in attenuation coefficient will result in a corresponding error of 40% in the determination of η_{bs}).

2. *Histochemical*

The tissue specimens, viz. bovine brain, liver and spleen, porcine liver and human liver were obtained at commercial slaughter houses or at post mortem (in the case of human liver), immediately after excision and brought to the laboratory for measurement within 30 min thereafter.

About 65% of the measurements were made on liver tissue. Results with brain and spleen, however, displayed no obvious tendencies to be different, in relation to the action of fixatives, from the results with liver. Fresh specimens were measured in phosphate-buffered saline (pH 7.3) and were placed in the fixing solutions immediately after (see results section for specimen dimensions) the acoustic measurements were completed. These were maintained in the fixative for at least two months, after which the acoustic measurements for the fixed specimens were carried out. Fixed specimens were measured in the solutions in which they were fixed. Where swelling of the tissue occurred during fixation the specimen was re-cut, so as to once again yield measurements through a known path length. Gaseous inclusions were removed from the specimens, before measurement, by manual manipulation

under the measurement fluid. All measurements were made at room temperature (20 ± 2 deg. centigrade).

RESULTS

The specimens for the attenuation measurements were slabs having plane, parallel major faces approximately 50 cm^2 in surface area and ranging in thickness from 1.5 to 3.0 cm.

Attempts were made to make measurements at the same positions on the tissue specimens both before and after fixing. Exact relocation of the original measurement positions was made difficult by the swelling and distortion of the tissues during fixation. Thus averages of measurements made in at least six different regions, each selected (on the criterion that attenuation was a relatively smooth function of frequency) for minimum phase cancellation artifact (Miller *et al.*, 1976), were used for each fresh and fixed specimen. For each specimen the average attenuation coefficient (α) and its standard deviation (σ), were determined at 44 frequencies in the range 1–7 MHz. Interpolation of these data to a smooth curve then allowed values of α to be obtained at frequencies 1–7 MHz, without presupposing that a particular frequency dependence law is obeyed. The mean percentage change occurring in the attenuation coefficient was then calculated, at each of the frequencies and for each specimen, as $(\alpha_2 - \alpha_1/\alpha_1) \times 100$; where α_1 and α_2 are the averaged attenuation coefficients before and after fixation respectively. Finally a group average, $\langle(\alpha_2 - \alpha_1/\alpha_1) \times 100\rangle$, was then computed for all specimens treated with a like fixative. Figure 1 shows the resulting mean values and standard errors (for the number of specimens shown) as a function of frequency for each fixative.

The specimens used for velocity measurements were cut and measured (using the above averaging procedure) in the same manner as the attenuation specimens. Figure 2 illustrates the changes observed in the speed of sound as a result of fixation. In the cases of formalin and ethyl alcohol, whose sound velocities differed appreciably from that of the tank water, the reference fluid (usually water) in the immediate vicinity of the specimen was replaced by a small inner-tank full of the fixative concerned. This procedure obviated the need to make corrections for the small amount of fixative

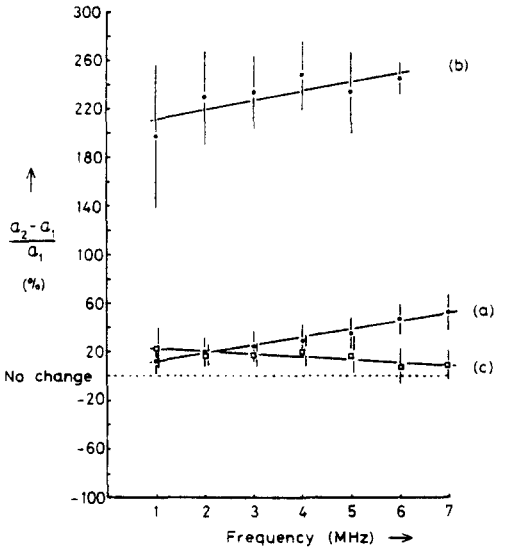

Fig. 1. Mean percentage change and standard errors in ultrasonic attenuation coefficients vs frequency for histochemical fixation in; (a) formalin (10 specimens), (b) ethyl alcohol (6 specimens), and (c) potassium dichromate (6 specimens).

present in the specimen holder but necessitated the measurement of sound velocity in the fixative fluids. Measurements of the speed of sound in ethyl alcohol were found to agree with published data (Giacomini, 1947) to within 0.2% whilst the value obtained for 4% formalin solution was within 0.2% of that to be expected from the result of Kjospes (1967), if a linear dependence on concentration is assumed.

The backscattering specimens were sculptured into right circular cylinders of approximately 1 cm radius and 5 cm length. As already mentioned, averaging was accomplished over 1000 discrete angular positions.

Figure 3 shows changes occurring in the average backscattering cross section per unit volume (defined and determined similarly to that for attenuation) for each of the fixatives. Here, and also in Figs. 1 and 2, it is seen that

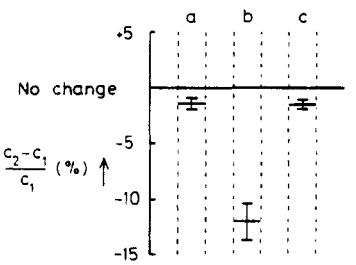

Fig. 2. Means and standard errors of percentage change in the speed of ultrasound for the three histochemical fixatives (as labelled in Fig. 1).

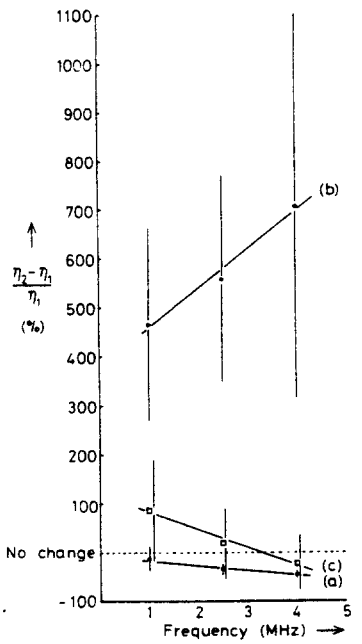

Fig. 3. Means and standard errors for percentage change in the average ultrasonic backscattering cross section per unit volume vs frequency for histochemical fixation in; (a) formalin (5 specimens), (b) ethyl alcohol (6 specimens), and (c) potassium dichromate (4 specimens).

ethyl alcohol is demonstrably inferior to the other two fixatives, as regards preservation of ultrasonic propagation properties.

It is important to note that computations of the backscattering cross section per unit volume require a knowledge of the attenuation and velocity within the scattering tissue. In the case of fresh tissue specimens these were obtained by direct measurements on flat slabs of tissue, from which the scattering cylinders were subsequently cut. This was clearly not possible for the fixed specimens and estimates of the parameters in this case were made by applying corrections, derived from Figs. 1 and 2, to the measurements made on the fresh specimens. In this way, it is believed that the results of Fig. 3 are, on average, reasonably accurate representations of the changes that occurred in the scattering parameter, independent of any changes that may have taken place in the attenuation and speed of sound. This method does, however, involve some uncertainty as to the value of the attenuation coefficient, to be used in equation 1, for fixed tissues: hence the relatively large standard errors in Fig. 3.

DISCUSSION

It seems reasonable to conclude, from the data presented above, that acoustic propagation properties can be preserved with an acceptable level of consistency by histochemical treatments. Though the formalin and potassium dichromate preparations are encouraging in this regard, experience in other aspects of histological technique suggests that pertinent inquiry could yield fixing mixtures which will be superior to the simple fixatives employed in this study, relative to their ability to produce preparations mimicking fresh *in vitro*, and possibly even *in vivo*, specimens.

There is a possibility that, because the tissue specimens to be fixed were quite large (though most were about 1.5 cm thick), differences between various fixatives might be expected in relation to their speed of diffusion and hence to their effectiveness in arresting central autolytic decay. Given, however, the time rates of change that have been observed for the acoustic propagation properties of non-preserved excised tissues (Bamber *et al.*, 1977; Frizzell, 1976; Shung and Reid, 1977) it is unlikely that the observed differences (Figs. 1–3) can be accounted for by such a mechanism. Similar comments also apply to possible variations in the time between excision and fixation (typically 3 ± 1 hr), which might have affected the consistency of the results.

Besides tissue preservation, fixation may have other benefits for the experimenter. It is commonly known that formalin fixation allows easier and more accurate cutting of specimens. The data obtained in the present study may also be used to provide a comparison of the fixatives in terms of the relative ease of handling of the fixed vs fresh specimens. It has already been mentioned that, at each of the frequencies (f) at which the average attenuation coefficient (α_f) was determined, a standard deviation (σ_f) was also computed. As a measure of the spatial variation of the attenuation coefficient, independent of frequency, the mean fractional standard deviation (MFSD) may be defined:

$$\text{MFSD} = \frac{1}{N} \sum_{f=f_1}^{f_N} \left(\frac{\sigma_f}{\alpha_f} \right) \qquad (2)$$

where N is the number of frequencies over which the summation is made. This quantity was computed for each of the tissue specimens in both fresh and fixed conditions. Figure 4 shows the values of the MFSD, plotted as fresh vs fixed, for the three

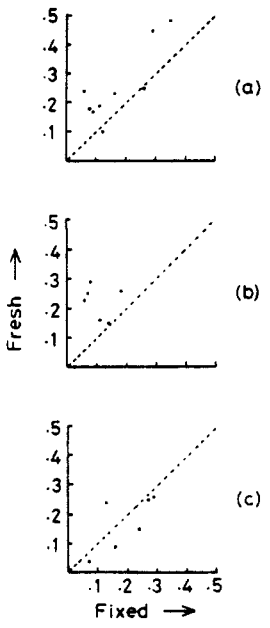

Fig. 4. Mean fractional standard deviations (MFSD—for definition see text) plotted fresh vs fixed, for the three histochemical fixatives; (a) formalin, (b) ethyl alcohol, and (c) potassium dichromate.

fixatives. It is seen that both formalin and ethyl alcohol appear to reduce the spatial variations of the attenuation coefficient while potassium dichromate does not. It is believed that these results simply reflect the differing properties of the fixatives with regard to handling the fixed specimens. Formalin and alcohol fixed tissues were rigid and could be re-cut more accurately, while fixation in potassium dichromate resulted in specimens that were no easier to handle than those in the fresh condition.

Finally, it is worth noting the potential value that this kind of data may have for gaining a better understanding of the interaction of ultrasound with the tissue structure. For example, in the present study it was at first thought that the increase in attenuation, resulting from fixation in ethyl alcohol, might be associated with the protein denaturation by dehydration—possibly akin to the effect observed by Pauly and Schwan (1971) when using heat to denature the proteins in liver. Whilst this may still, in part, be true, it is not consistent with the extremely large increase in the backscattering coefficient that was observed for alcohol fixation. A more plausible explanation is that, whatever the predominant scattering structures are, they are usually suspended in a watery medium. When this is replaced by alcohol, which has a much lower sound velocity (1170 ms^{-1} as compared to about 1485 ms^{-1} for physiological saline at 20°C), the impedance mismatch between the scatterers and the surrounding medium is increased. This would result in a large increase in the backscattering coefficient and a consequent (although smaller) increase in the attenuation coefficient. At least two ways to test this hypothesis may be envisaged; (a) alcohol has a temperature coefficient of velocity that is of opposite sign to that of water and tissue, thus scattering (and possibly attenuation) by alcohol. fixed tissues might be expected to rise rapidly with increasing temperature, (b) simply replacing the alcohol, either partly or completely, by water should allow the scattering (and attenuation) to be reduced. The latter phenomenon has in fact been observed for attenuation, on the microscopic scale, in plant tissue (Yuhas, 1978). Clearly much work has to be done in this field; many more fixatives and tissue types should be investigated over wider ranges of frequency, temperature, etc. A particularly interesting aspect may be the effects of histological fixation on the properties of acoustically anisotropic tissues such as muscle. Here possible differences, with respect to fibre orientation, have been indicated (McNeely and Noordergraaf, 1977), and have since been quantified for bovine skeletal muscle (Nassiri, 1978). A 30% increase in the attenuation coefficient was observed, due to formalin fixation, when measured perpendicular to the line of the muscle fibres, while no significant change was observed along the fibres.

Acknowledgements—This work was supported by grants from the Medical Research Council.

For F. Dunn, the work reported in this paper was undertaken during the tenure of an American Cancer Society—Eleanor Roosevelt—International Cancer Fellowship awarded by the International Union Against Cancer.

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