Ultrasonic attenuation and velocity properties in rat liver as a function of fat concentration: A study at 100 MHz using a scanning laser acoustic microscope

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This study examines the extent to which ultrasonic attenuation coefficients and velocity properties change between normal and fatty rat liver. The view of this problem is toward the application in clinical medicine in the future. Fatty livers were produced in rats by feeding them alcohol diets in liquid form. The animals were sacrificed and the fat concentration of the liver specimens determined. The fat concentration varied from 2.5% to 16.8% wet weight. The ultrasonic attenuation coefficient and velocity properties in 28 specimens were measured at 100 MHz with the scanning laser acoustic microscope (SLAM). Regression analysis was applied to the liver's ultrasonic propagation properties as a function of fat concentration. The results show that the attenuation coefficient increases at a rate of 1.08 dB/mm/% fat and the velocity decreases at a rate of 2.3 m/s/% fat as the fat concentration increases.

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INTRODUCTION

Fatty liver disease from high alcohol consumption is common in today's society. Early detection of this seriously damaging disease by a nondestructive technique could open up many new areas of interest for medical imaging, especially in the preclinical stages of the disease.

Ultrasonic attenuation and velocity properties of rat liver and abdominal wall fat specimens were measured using the 100-MHz scanning laser acoustic microscope (SLAM, Sonomicroscope 100®, Sonoscan Inc., Bensenville, IL). The goal is to determine the variation of the ultrasonic propagation properties as a function of the fat concentration. If the properties of normal and fatty liver differ, their measurement could make a definite contribution to the early detection and treatment of fatty liver disease.

The content of the paper is as follows. First the diets, which caused the creation of fatty livers in rats, are reported. Then the specimen handling, determination of the fat concentration, and the acoustic measurement techniques are explained. In Sec. III, the acoustic propagation properties are analyzed with a least-squares technique as a function of fat concentration. In Sec. IV the data are compared to those of a preliminary study and further discussed in relation to alterations in fat and other liver components due to diet changes.

I. CREATION OF FATTY LIVER IN RATS AND SPECIMEN PREPARATION

Twenty eight male Sprague–Dawley rats and three separate diets were used in this study. Table I details the number of liver specimens and the type of diet fed in two separate experiments, each lasting four weeks. In the first, 16 rats were divided into four groups among diets 1 and 2 (see Tables II and III). The critical difference between diets 1 and 2 is the amount of fat, where about 19% of the diet comes from fat in diet 2 and about 8% of the diet from fat in diet 1. In the alcohol groups, absolute ethanol was isocalorically substituted for sucrose to provide 30% of the total calories. For the second experiment 12 rats were placed on the Lieber–DeCarli diet,[1] which contained 20% casein, 54.5% dextrin–maltose, 18.7% fat, and either 0% or 36% of total calories as ethanol. The Lieber–DeCarli diets differ from diets 1 and 2 in their carbohydrate makeup; they are also high in fat.

After four weeks on the respective diets, the animals were sacrificed, their livers removed, and a small portion cut off of each for this study. To prevent cell lysis, the pieces were quick frozen in dry ice and acetone solution and stored at $-20\,^{\circ}$C until they were ready to be examined. In general,

TABLE I. Details of the number of animals and their diet for experiments 1 and 2. Diet 1 is listed in Table II, diet 2 is listed in Table III, and the Lieber–DeCarli (LD) diet is described in the text.

Number of animals	Diet	Ethanol
Experiment 1		
4	1	0%
4	1	30%
4	2	0%
4	2	30%
Experiment 2		
7	LD	0%
5	LD	36%

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TABLE II. Diet 1 composition. Casein, mineral mix, and vitamin mix were supplied by Teklad Test Diets, Madison, WI.

Component	% by weight	% of calories
Casein (vitamin-free)	20	19.1
Fat (corn oil)	8	16.8
Mineral mix (mineral mix U.S.P. XVII)	4	0.0
Vitamin mix	1	0.0
Sucrose	67	64.1
	100	100.0

the specimens remained frozen from 2 weeks to 2 months. Different portions of the same liver specimen, and not necessarily the same lobe, were used for the two SLAM measurements and for the fat concentration determinations.

The liver's fat concentration was determined using the technique described by Folch.[2] The fat concentration is reported in terms of percentage of fat on a wet weight basis of liver with an accuracy better than $\pm 0.5\%$. The study was blind in that when the ultrasonic propagation properties were measured, knowledge of either the specific diet or liver fat concentration was not known.

Figure 1 is a closeup view of the experimental arrangement on the microscope stage surface. A small amount of distilled water was placed directly on the sonically activated, fused silica portion of the stage (2.5×5.1 cm). Water served as an acoustic coupler between the stage and the plastic slide on which the specimens were placed.

Four specimens were cut out from the inside of the frozen, then thawed section of liver. Care was taken to avoid obvious inhomogeneities such as venule or arterial clusters. These were then prepared by cutting with a two-razor-blade technique under magnification to a thickness just slightly greater than and placed within a metal spacer on the plastic slide. Normal saline served as the acoustic coupling medium on the plastic slide. Four spacers with thicknesses of 370, 550, 740, and 920 μm were used. A partially mirrored coverslip was placed on top of the biological specimen and supported by this spacer, which was used to keep a uniform and known specimen thickness and to prevent the coverslip from compressing or distorting the tissue. This technique kept the uncertainty in the specimen thickness small, viz., around $\pm 10\,\mu$m for all thicknesses. Therefore, the percentage uncertainty ranged from $\pm 1\%$ to $\pm 3\%$.

The same tissue handling procedure was also used for the fat specimens which were taken from near the rat's abdominal wall. The same considerations were also applied for the thickness uncertainty.

TABLE III. Diet 2 composition. Casein, mineral mix, and vitamin mix were supplied by Teklad Test Diets, Madison, WI.

Component	% by weight	% of calories
Casein (vitamin-free)	22.4	19.1
Fat: olive oil 72% safflower oil 19% corn oil 9%	18.7	35.0
Mineral mix (AIN-76)	4.0	0.0
Vitamin mix	1.0	0.0
Sucrose	53.9	45.9
	100.0	100.0

FIG. 1. A picture of the liver specimen placement on the stage of the SLAM. The inside and outside diameters of the metal spacers are 6.4 and 13 mm, respectively.

II. ATTENUATION COEFFICIENT AND VELOCITY MEASUREMENT TECHNIQUES

The velocity and attenuation coefficient measurement techniques have been reported in detail[3–5] and thus are described only briefly. All measurements were made at room temperature within 3 h from the time the liver specimen was thawed.

The attenuation coefficient measurement technique[3] utilized the insertion loss method, which involves the comparison of the received signal amplitude with and without a specimen of known thickness in the sound path. Four values of insertion loss (IL) were recorded for each of the four thick-

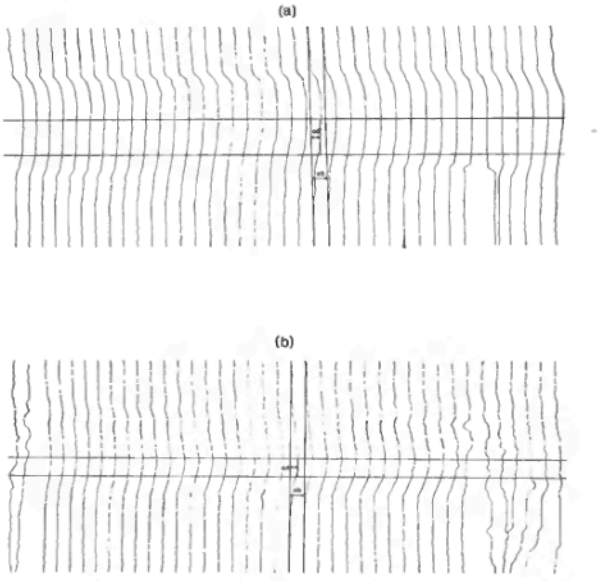

FIG. 2. Computer processed interferograms of two 370-μm-thick rat liver specimens of fat concentrations of (a) 3.26% and (b) 16.81%.

nesses. The slope of the IL versus thickness curve is determined by a least-squares analysis and yields the attenuation coefficient. The insertion loss measurement sensitivity is 0.2 dB within the signal amplitude range employed in the study and its reproducibility is $\pm 4\%$. Considering the specimen thickness range (370–920 μm) and its uncertainty ($\pm 1\%$ to $\pm 3\%$), the uncertainty of the attenuation coefficient is $\pm 5\%$.

The velocity measurement technique[4,5] utilizes the SLAM's interferometric mode, which provides the relative phase change of the wave after it has propagated through the specimen. This is illustrated in Fig. 2, which shows two computer processed interferograms. Figure 2(a) and (b) is an interferogram example of liver specimens for low (3.26%) and high (16.81%) fat concentrations, respectively.

The equation for determining the ultrasonic velocity is[6]

$$C_x = (C_0/\sin\theta_0)\sin\{\arctan[(1/\tan\theta_0) - (N\lambda_0/T\sin\theta_0)]^{-1}\}, \quad (1)$$

where C_0 is the velocity of sound in saline (1507 m/s), θ_0 is the angle of the beam from the normal in saline (10.9°), λ_0 is the wavelength of the sound in the surrounding medium, T is

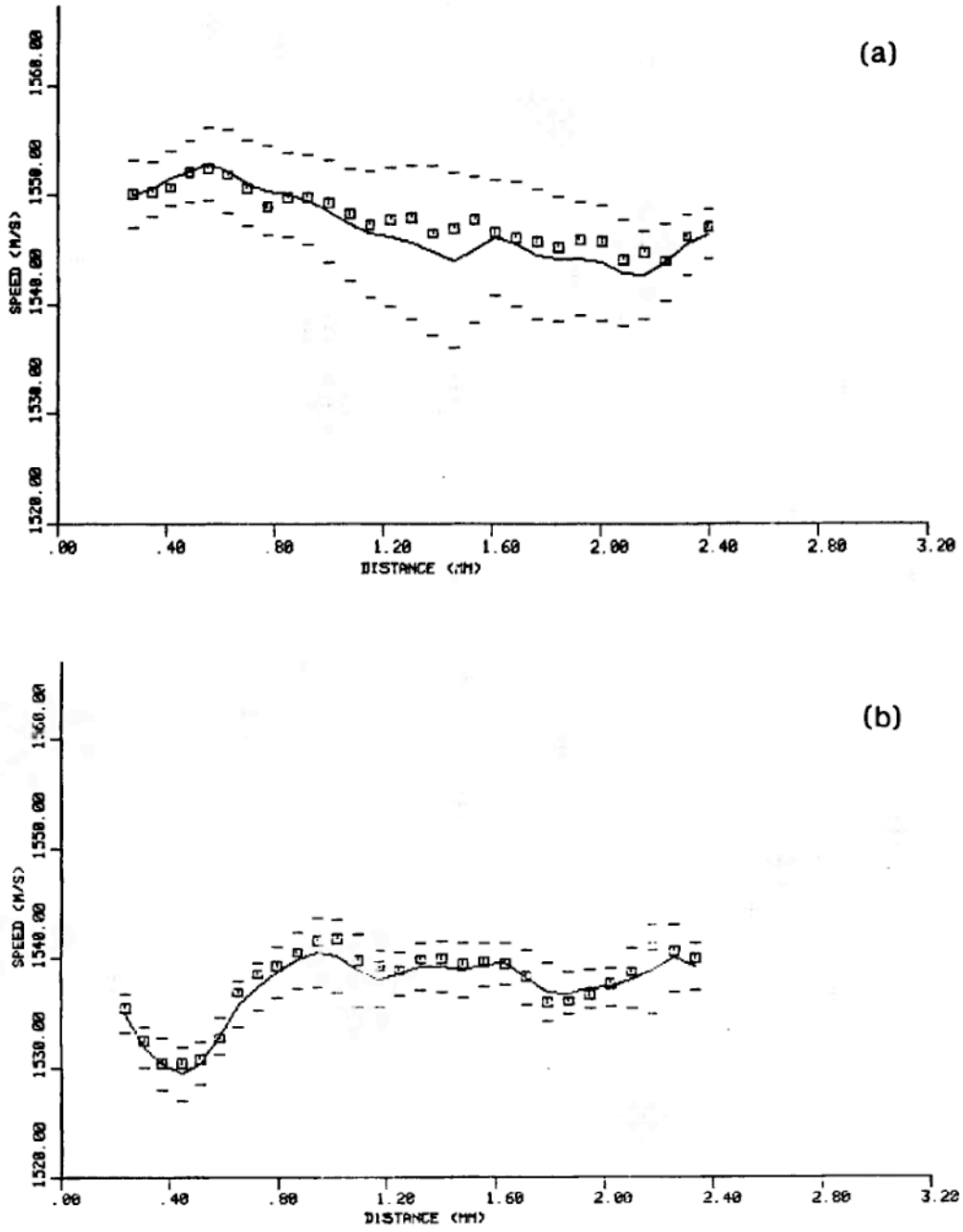

FIG. 3. Computer processed output of the velocity values along the specimens using the two interferograms from Fig. 2; (a) corresponds to Fig. 2(a) and (b) to Fig. 2(b). ⊐, distance showing $\pm$ standard deviation; □, median value; -------, curve as plotted through the mean values.

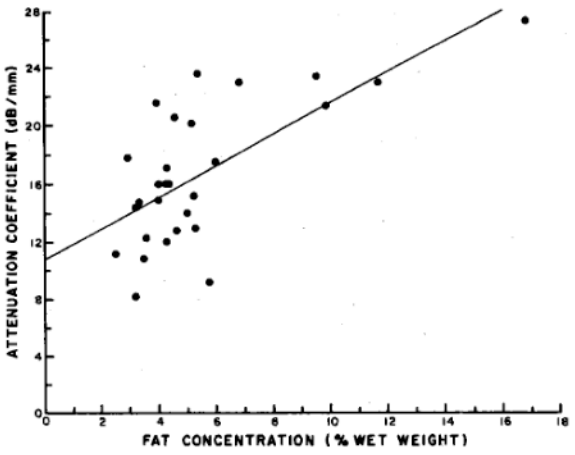

FIG. 4. Ultrasonic attenuation coefficient at 100 MHz as a function of the rat liver's fat concentration. The solid line represents the least-squares fit to all of the data [see Eq. (2)].

the thickness of the specimen, and N is the normalized lateral fringe shift (ad/ab, see Fig. 2). For each interference line, a value of ad is calculated for each data point within the limits indicated by the two horizontal lines in Fig. 2. Within these limits is the specimen of known thickness. Thus a distribution of C_x values from Eq. (1) is calculated per interference line. From this distribution the velocity's mean, medium, and standard deviation are calculated.

Figure 3 graphically demonstrates examples of the processed velocity data from the interferograms of Fig. 2. The mean velocity and its standard deviations, calculated from the C_x distribution, are shown for each of the vertical interference lines. The continuous line in Fig. 3 represents the mean values and the horizontal dashes above and below the mean value data points are the $\pm$ 1-s.d. boundaries. The rectangular symbols show the median values of the velocity distributions.

The velocity value reported for a single specimen at a specific thickness in this paper is determined as the mean from the approximately 35 mean velocity values along the specimen, as shown in Fig. 3. Two parameters from Eq. (1) directly affect the measurement uncertainty, viz., N and T. If one assumes that the uncertainty of these are $\pm$ 10% and $\pm$ 3%, respectively, then this results in an uncertainty for C_x of $\pm$ 0.3%.[5]

III. RESULTS

The attenuation coefficient and velocity results are graphically represented in Figs. 4 and 5, respectively. The attenuation coefficient as a function of fat concentration for the data were fit with a least-squares analysis to yield

$$A = 10.7 + 1.08F, \tag{2}$$

where A is the ultrasonic attenuation coefficient in dB/mm and F is the fat concentration in % wet weight. The correlation coefficient of the fit is 0.69 and the mean value of the absolute deviation from the line is about $\pm$ 3 dB/mm. Equation (2) is plotted in Fig. 4.

Four velocity measurements were performed for each specimen, one for each of the thickesses. Figure 5 details the 112 velocity values for the 28 specimens as a function of their fat concentrations. The least-squares fit lines for each of the specimen thicknesses are shown in Fig. 5 and the results are listed in Table IV. The slopes and intercepts for the four thicknesses are nearly the same. The maximum extent of the four least-square lines over the liver's fat concentration up to 17% is within $\pm$ 0.3% of the least-squares fit line determined by using all 112 velocity values, that is,

$$V = 1557 - 2.3F, \tag{3}$$

where V is in m/s. The correlation coefficient is $-$ 0.61 and the mean value of the absolute deviation of the measured points from the line is about $\pm$ 8 m/s. Equation (3) is represented as the solid line in Fig. 5.

Pure fat from near the abdominal wall was measured in two specimens from the same rat. The ultrasonic attenuation coefficients were determined from the insertion loss measurements of the four thicknesses to be 44.7 and 54.5 dB/mm. The velocity values were 1456 and 1467 m/s; here, only the thinnest specimen thickness of 370 μm was used.

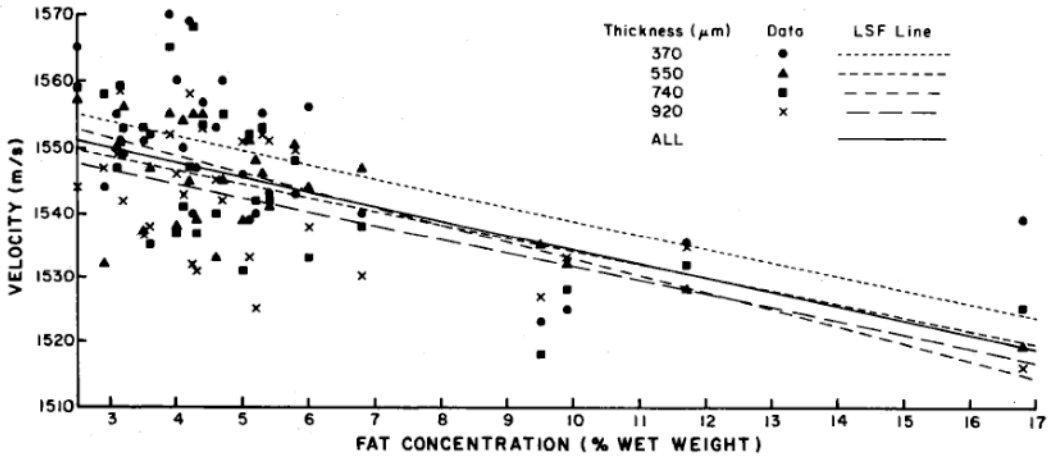

FIG. 5. Ultrasonic velocity as a function of the rat liver's fat concentration. The four different dashed lines represent the least-squares fit to the velocity data for each of the thicknesses. The solid line is the LSF for all of the data [see Eq. (3)].

TABLE IV. Least-squares fit analysis of the velocity data as a function of fat concentration. Specimen thickness is used as the criteria for subgrouping the velocity data. For each of the 28 liver specimens, there were four velocity value determinations, one for each thickness.

Specimen thickness (μm)	Number of velocity values	Intercept (m/s)	Slope [(m/s)/% fat]	Correlation coefficient
370	28	1560	−2.2	−0.59
550	28	1555	−2.1	−0.68
740	28	1560	−2.7	−0.66
920	28	1553	−2.1	−0.62
Combined	112	1557	−2.3	−0.61

IV. DISCUSSION

Alcohol consumption can lead to an increase in liver size, or hepatomegaly, due to an increase in hepatocyte volume. Although fat accumulation accounts for a large proportion of this increase, protein and water also accumulate within the cell.[7,8] Alcohol consumption also causes ultrastructural alterations, such as mitochondrial enlargement and proliferation of the smooth endoplasmic reticulum.[9–12]

In this study the trends of the ultrasonic attenuation coefficient and velocity data over an extended fat concentration range are analyzed with a least-squares analysis, which shows a positive slope for the attenuation coefficient [Eq. (2)] and a negative slope for the velocity [Eq. (3)] as a function of increasing fat concentration.

Table V lists the ranges and mean values for the fat concentration, attenuation coefficient, and velocity based upon the grouping by dietary condition. The low fat diet 1 did not produce a very high concentration of fat in the livers, which was also the case for the high fat diet 2. With both of these diets, the ethanol-fed rats developed just slightly greater fat concentrations in the liver than did the nonethanol-fed rats. Here, the mean attenuation coefficient for the ethanol-fed rats ranges from 13% to 16% higher when compared within each of these diets, which is greater than the measurement uncertainty. On the other hand, the mean velocity, while lower for the ethanol-fed rats, was well within the measurement uncertainty.

The rats fed the Lieber–DeCarli diet produced a greater increase in fat concentration for both the ethanol-fed and nonethanol-fed animals, as compared to diets 1 and 2. The mean attenuation coefficient was 60% higher and the mean velocity was almost 1% lower in the livers of the ethanol-fed rats for this diet group. These ultrasonic property changes are well in excess of their measurement uncertainties.

For the nonethanol-fed rats from the three diets, the mean attenuation coefficients are within $\pm 9\%$ of each other, which is in excess of the $\pm 5\%$ measurement uncertainty. The fat concentration ranges for these livers were different, but the trend of increasing fat with increasing attenuation coefficient is not followed. This could suggest that diet components, other than ethanol, have an effect upon the liver. The three mean velocities for the nonethanol-fed rats are within $\pm 0.23\%$, which can be accounted for by measurement uncertainty. This suggest that there is no measurable difference of the livers to which velocity is sensitive; however, this does not appear to be the case for the attenuation coefficient for these three dietary conditions of nonethanol.

There are two sets of dietary conditions which allow for the comparison of the ultrasonic properties with similar fat concentrations. Comparing the ethanol-fed rats of diet 1 with the nonethanol-fed rats of diet 2 shows that the differences in both of the ultrasonic propagation properties are well within their measurement uncertainties. However, this is not the case for comparing the ethanol-fed rats of diet 2 with the Lieber–DeCarli, nonethanol group. Here, there is a 16% decrease in the mean attenuation coefficient from the ethanol-fed rats of diet 2 even though there is only a slight increase in the fat concentration; it should also be observed that this is opposite to the general trend noted until this point. Again, these general comparisons between the means of two different experiments suggest that the diet may be affecting the liver in such a way that fat is not the only determinant in changing the ultrasonic properties.

To further explore this point, a very high fat content tissue was evaluated, viz., the rat abdominal fat pad. Substituting into Eqs. (2) and (3), the mean values of A and V obtained for the rat abdominal fat (49.6 dB/mm and 1462 m/s) yield fat concentrations of 36% and 42%, respectively. These values are lower than the normal amounts of fat in fat tissue, that is 50% to 86%.[13] The comparison, of course, assumes that the liver fat can be represented by the abdominal fat pad. However, extrapolating Eqs. (2) and (3) to very

TABLE V. Maximum range and mean value (in parentheses) for the fat concentration, attenuation coefficient, and velocity based upon the grouping by dietary condition.

Diet	Ethanol concentration (%)	Number of specimens	Fat concentration (%)	Attenuation coefficient (dB/mm)	Velocity (m/s)
1	0	4	2.5–3.3 (3.0)	8.2–17.8 (13.0)	1545–1556 (1550)
1	30	4	3.5–4.0 (3.8)	10.8–21.5 (15.1)	1540–1561 (1548)
2	0	4	3.2–4.3 (4.0)	14.4–17.1 (15.6)	1547–1555 (1553)
2	30	4	4.3–5.4 (4.8)	12.0–23.6 (17.6)	1539–1544 (1542)
LD	0	7	4.3–6.0 (5.2)	9.2–20.1 (14.8)	1539–1552 (1546)
LD	36	5	6.8–16.8 (11.0)	21.3–27.3 (23.6)	1525–1539 (1532)

high fat concentration values results in a much higher extrapolated attenuation coefficient and a much lower extrapolated velocity than the measured ultrasonic propagation properties for fat. Thus is appears that another constituent is necessary to more fully explain the ultrasonic behavior. This factor could be water, which increases in liver as a result of alcohol[7] and is the other major constituent of the abdominal fat pad. Water would have the effect of decreasing the attenuation coefficient and increasing the velocity, thus bringing these extrapolated values closer to the measured range for fat.

In an earlier preliminary study,[4] in which rats were placed on an alcohol diet (diet 1, Table II) to produce fatty livers, the fat concentrations ranged from 2.6% to 5.1%. Here, both the ultrasonic attenuation coefficient and velocity of the 29 liver specimens were measured with SLAM. Because of the narrow fat concentration range from the preliminary study, as compared to that obtained in this study, block averaging of the attenuation coefficient and velocity data for the two studies has been employed in order to aid in their comparison.

The attenuation coefficient data were block averaged based upon 1% increments of the liver's fat concentration (see Table VI). For the lowest three liver fat concentration ranges (2% to 5%), comparison between the block averaged data sets from the two studies shows that there is essentially no difference in the attenuation coefficient, that is, in the range of 13–16 dB/mm at 100 MHz. Note that if the attenuation coefficient data within the fat concentration range from 2% to 5% are considered essentially the same and thus can be averaged together, then the 29 data samples from the preliminary study[14] yield a mean attenuation coefficient of 15.4 dB/mm, and the 16 data samples from this study yield 14.8 dB/mm, which is within the $\pm$ 5% uncertainty for the attenuation coefficient measurements.

Comparing the velocity data is more difficult because two different measurement techniques were employed; the preliminary study yielded only maximum (C_{max}) and minimum (C_{min}) velocity values for each specimen. Table VII is a summary of the velocity data that has been block averaged for each study based upon 1% increments for the liver's fat concentration. For the preliminary study, the mean values for both C_{min} and C_{max}, along with their mean difference, $C_{max} - C_{min}$, are provided for each fat concentration range.

TABLE VI. Summary of block averaging based upon the liver specimen's fat concentration for the ultrasonic attenuation coefficient for two studies.

Fat concentration	Attenuation coefficient (dB/mm)[a]	
Range %	Mravca[14]	This study
2.0–2.9	13.5(3)	14.5(2)
3.0–3.9	16.0(16)	13.7(6)
4.0–4.9	15.0(9)	15.7(8)
5.0–5.9	15.8(1)	14.2(6)
6.0–6.9		20.3(2)
9.0–9.9		22.3(2)
11.7		22.9(1)
16.8		27.3(1)

[a] The numbers in parentheses are the numbers of the specimens included for averaging.

C_{min} and C_{max} were determined by manually evaluating a number of interference lines for each specimen and choosing the minimum and maximum velocity values. For this study, the mean values of C_x for each fat concentration range were determined as described in Sec. II. Also available from this analysis was the minimum and maximum values and thus $C_{min} - C_{max}$, which is used for comparison with the preliminary study. If one were to assume that a mean velocity value for the preliminary study could be the mean of the C_{min} and C_{max}, then the difference between the means from the two data sets for each fat concentration range would be within 8 m/s for the lowest three fat concentration ranges. For the liver velocity data within the 2% to 5% fat concentration range, the 28 data samples from the preliminary study yield means of C_{min} and C_{max} of 1533 and 1578 m/s, respectively. The mean velocity for the 16 data samples in this study over the same fat concentration range yields a mean of 1549 m/s, which is just slightly lower than 1556 m/s, the mean value of the C_{max} (1578 m/s) and C_{min} (1533 m/s) obtained in the preliminary study. This difference is just slightly greater than the $\pm$ 0.3% uncertainty for the velocity measurements although the methods employed and the procedure for reporting the data were both somewhat different.

Previous work has shown that the attenuation coefficient for fresh bovine liver, measured with the SLAM, to be 13.4 $\pm$ 1.7 dB/mm at 100 MHz.[15] There are no known additional reports of the ultrasonic attenuation coefficient in liver tissue, or in other types of tissue, at or around 100 MHz. In this study, the rat liver attenuation coefficient values in the low fat concentration range are from 13–16 dB/mm, which are in agreement with each other. Velocity values for sheep and cat liver at 100 MHz have been reported to be 1565 and 1567 m/s, respectively.[16] These values are somewhat greater than our values in the low fat concentration range by about 1%. Fresh bovine liver measured at lower frequencies has yielded velocities in the range 1545–1641 m/s[17,18] and can also be compared to the current data because reports show that the little difference exists in ultrasonic velocity values measured at various frequencies in the 1- to 100-MHz range.

A clinical trial with 14 patients suggested a correlation between the estimated ultrasonic attenuation coefficient and liver disease.[19] The attenuation coefficient was estimated from the reflected ultrasonic signals from within the liver; the liver disease was based mainly upon biopsy. The patients with the fatty livers represented some of the highest values of the attenuation coefficient among the various diseases' data.

Excised normal and fatty human liver samples showed an increase in the ultrasonic attenuation coefficient (via an insertion loss type measurement) in the 1- to 5-MHz frequency range as a function of an increase in fat concentration.[20] The six fatty liver specimens (9.0% to 35.5% fat concentration) had a mean ultrasonic attenuation coefficient about 35% greater than the eight normal specimens (3.6% to 3.8% fat concentration).

The attenuation coefficient and velocity properties have been quantitatively evaluated in the same liver specimens as a function of fat concentrations in only one other report.[21] An increased attenuation coefficient was reported in 28 excised human liver specimens as a function of fat concentra-

TABLE VII. Summary of block averaging based upon the liver's fat concentration for the ultrasonic velocity for two studies. The numbers in parentheses are the numbers of the specimens included for averaging

Fat concentration range	Mravca[14]			This study	
(%)	C_{min} (m/s)	C_{max} (m/s)	$C_{max} - C_{min}$ (m/s)	C_x (m/s)	$C_{max} - C_{min}$ (m/s)
2.0–2.9	1525(3)	1582(3)	57	1551(2)	24
3.0–3.9	1537(16)	1579(16)	42	1550(6)	14
4.0–4.9	1529(9)	1575(9)	46	1547(8)	18
5.0–5.9	1537(1)	1589(1)	52	1545(6)	15
6.0–6.9				1541(2)	20
9.0–9.9				1528(2)	13
11.7				1533(1)	7
16.8				1525(1)	23

tion. This relation becomes stronger as the ultrasonic frequency increases from 2 to 7 MHz. At 2 MHz, there was no correlation of the attenuation coefficient with fat, but at 7 MHz, the attenuation coefficient increased as a function of fat concentration with a slope of 0.0091 dB/mm/% fat. At 100 MHz, from Eq. (2), this dependency is 0.083 dB/mm/% fat. It is interesting to observe, and perhaps speculate, that this measured dependency is not only frequency dependent but also that the dependency may be approximately proportional to frequency.

In terms of the velocity dependency upon fat concentration, within the 2–7 MHz frequency range, this dependency has been reported[21] to be -1.8 m/s/% fat. From Eq. (3), this dependency is -2.3 m/s/% fat, which again suggests that such a dependency is a function of frequency and a bit greater at higher frequencies. This report[21] supports the proposed hypothesis that as the fat concentration increases in liver tissue, the attenuation coefficient increases and the velocity decreases. Further, while only suggestive, these observations[21] tend to support the idea that the attenuation coefficient may be more strongly dependent upon increasing fat concentration than that of velocity, both in terms of its frequency dependency and its sensitivity to detect changes in the liver. The implications of these observations are not yet fully appreciated. Clearly, further ultrasonic propagation property measurements are needed to evaluate more quantitatively their dependency upon the tissue's constituents.

Note added in proof: The fat pad's fat concentration was determined using the technique described by Folch[2] to be 79.3%.

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