Carbon Tetrachloride Induced Changes in Ultrasonic Properties of Liver

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Abstract—**This paper presents a study of rat livers excised at 1, 5, and 24 h after injection of the toxic agent carbon tetrachloride ($CC\ell_4$). The $CC\ell_4$ damage results in a swollen, fatty liver at 24 h postinjection, with some necrosis localized in centrilobular regions. Measurements of ultrasonic absorption, attenuation, and backscatter were obtained over a frequency range of 1–11 MHz, and the values were compared to those obtained from a control group of rats who received no injections. Significant changes in absorption and attenuation were observed as a function of time; however, little change in backscattered signal strength was noted. These data are presented along with a description of structural and biochemical changes that are typical of the rat liver response to $CC\ell_4$ poisoning. The results may provide an explanation for the variation among preliminary attenuation estimates obtained from humans, as reported by different investigators.**

INTRODUCTION

ULTRASONIC measurements can characterize certain pathological changes in soft tissue. Initial experimental evidence for this has come from laboratory studies on excised tissue samples, although some results were obtained *in vivo*. Early attention was given to ultrasonic attenuation and backscatter changes in myocardial infarcts [1]–[3] and ophthalmological tissues [4], and recent work has included larger organs or tissues including the liver and breast [5]–[14]. Despite the encouraging, early *in vitro* results, the clinical applications of quantitative diagnosis are developing slowly because of technological and theoretical reasons. Clinical measurements of the true magnitude and frequency dependence of ultrasonic attenuation coefficients of tissue [5], [6] or of a "slope" parameter related to attenuation [8], [13] all require optimal design of hardware, and the use of signal processing algorithms based on necessarily complex acoustic theory [5], [6], [15]–[17]. Other problems concern unresolved fundamental issues of ultrasonic interaction with tissues. For example, in scattering research, the contributions of density and compressibility fluctuations, the scale of inhomogeneities, and the applicability of Born and Rytov approximations are under active investigation [12], [18]–[23]. In attenuation work, the issues of the contributions of absorption and scattering, and the identification of major underlying mechanisms and appropriate relaxational (or other) models, still receive close attention [24]–[31].

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To address some of these issues, we undertook a study of ultrasonic properties of rat livers affected by carbon tetrachloride poisoning. This model was chosen for a number of reasons. First, $CC\ell_4$ toxicity has been extensively studied in the past few decades, and is known to affect the liver biochemistry and structure, leading to a condition characterized rather simply as "fatty liver" [32]–[36]. Thus, an appreciable base of knowledge exists concerning the damage and repair sequences of $CC\ell_4$ toxicity. Furthermore, the condition has some relevance to analogous fatty liver conditions in human livers. For these reasons, the rat liver absorption, attenuation, and backscatter coefficients were studied as a function of time postinjection. The changes in measured ultrasonic parameters are compared to the biochemical and structural changes which occur as a function of time, in order to provide data relevant to fundamental issues of tissue-ultrasonic interaction. The $CC\ell_4$ liver study also demonstrates parameter variations which may be observed in clinical studies underway in our laboratory and others.

THEORY

A. Interaction of Tissue and Ultrasound

A brief review of basic phenomena is given here to assist in the interpretation of results. Ultrasonic attenuation in normal soft tissues, for the most part, increases nearly linearly with frequency over the 1–10 MHz decade commonly used in diagnostic ultrasound [41], [42]. The primary mechanism for the absorption of sound in biological material appears to lie at the macromolecular level [26], [27], [30], [37], [38] where protein–protein interactions or protein–water interactions contribute relaxation processes which cover a broad range of relaxation frequencies. Collagen in particular has been identified as a protein which increases ultrasonic attenuation and absorption [27], [28], [40]. The role of fats or triglycerides in total tissue attenuation is less clear, as different fatty tissue attenuation coefficients have been reported as being either higher or lower than normal values for soft tissue such as liver [39]–[42].

Mathematical models of absorption exist which can generate the nearly linear-with-frequency increase in attenuation which is observed in soft tissues. In particular, a model employing a continuous distribution of relaxation mechanisms over a finite but wide band of frequencies, with appropriately shaped distribution functions, can pro-

duce a resulting absorption curve which increases as the square of frequency at low frequencies, then becomes linear with frequency within much of the band of relaxation frequencies, then tends towards a constant value at very high frequencies [30], [43]. Unfortunately, values of absorption or attenuation measured within 1–10 MHz are usually insufficient for a meaningful "inverse solution," where the form of the underlying relaxation distribution would be determined from the measured attenuation data [43].

Other mechanisms are capable, at least theoretically, of producing attenuation versus frequency curves which mimic those observed in tissue. For example, the relative motion between a viscous medium and suspended particles of different density has been studied [29], [31], [37], [43]. Given a specialized distribution of sizes and physical parameters of spherical particles in a viscous medium, an absorption versus frequency curve can be calculated which shares some of the characteristics of the multiple relaxation models mentioned previously, such as a linear increase in attenuation over a band of frequencies. The relative motion model may prove useful in special cases such as microcalcifications in breast tissue, but is not considered to be a generally operative mechanism in soft tissues [27], [37].

Another important ultrasonic property of tissues is the scattering of longitudinal waves as a function of angle and frequency. A theory based on small fluctuations of density and compressibility within a medium has been applied to explain observations [15], [18], [19], [44].

The pattern of scattered pressure is shown to have a Fourier transform relation to the structure of inhomogeneities within a sample volume of the medium [19], [44]. Similarly, for random media, the ensemble averaged scattered intensity pattern has a Fourier transform relationship to the correlation function of the medium inhomogeneities. Recent results indicate that calf liver is comprised of weak scatterers having a correlation length on the order of 1 mm [18]. If scattering were produced by small, micron-sized components, the scattering would tend to be omnidirectional and increase as frequency to the fourth power; however, calf liver results show a tendency towards forward scattering, increasing only as frequency to the 1.4 power [18].

The total power scattered per unit volume by liver is not an appreciable fraction of the incident power between 1 and 7 MHz. Scattering is therefore a minor contributor to total attenuation; absorptive processes dominate the loss mechanisms [18], [24]. The role of proteins in scattering has been studied, and in particular, collagen has been shown to increase the backscattered signals from tissue [11], [22].

B. Effects of Carbon Tetrachloride

Research on $CC\ell_4$ toxicity has been carried on for many decades as a classic model for the study of drug sensitivity. Thus, the biochemical mechanisms and damage sequences have been extensively documented [32]–[36]. A brief description of some major effects are given here, following principally the works of Recknagel [32]–[34]. For mature rats and other mammals, the primary toxic effects of $CC\ell_4$ are localized to the liver as metabolism produces cleavage of the $CC\ell_3$–$C\ell$ bond, which in turn results in major pathological damage to the endoplasmic reticulum of the cells. Here, lipid peroxidation (akin to rancidity in fatty foods) is thought to be the most important immediate consequence of the lethal clevage. These events are the beginning of a complex maze of biochemical and structural changes. At doses around 0.25 ml $CC\ell_4$/100 g body weight in adult rats, the toxicity will result in a "fatty liver" within 24 h. At this time postinjection, the resulting necrosis and fat deposition are not uniformly distributed throughout all cells, but instead vary predictably with position within liver lobules. Table I outlines some of the biochemical and structural changes that occur as a result of $CC\ell_4$ poisoning in rats. The list is not exhaustive; however, an attempt has been made to summarize the effects which are more commonly discussed in the literature [32]–[36]. The intervals of Table I concur with ultrasonic measurement times, although a detailed description of the biochemical changes would require a finer time increment. As a general statement, however, the fatty liver produced at 24 h can be thought of as being swollen with increased fat and water content compared to normal livers. A fine dotted appearance can be observed by the unaided eye, as individual lobules which have a typical size of approximately 1 mm generally exhibit necrotic central regions, "swollen" midzonal regions composed of vacuoles which do not contain fat or glycogen, and peripheral regions where cells which are either unaffected or contain fat droplets.

METHODS

Young Wistar male and female rats, weighing approximately 200 g, were used in this study. Rats were injected with a dose of 0.25 ml $CC\ell_4$/100 g weight, administered interperitoneally. Animals were killed by cervical dislocation at 1 h, 5 h, and 24 h postinjection. The numbers of animals involved were four, four, and five, respectively, in each group. An additional group of three received no injections and were used as controls. Whole livers were excised and immediately placed in chilled physiological saline. Measurements were made at 22°C within 1–5 h of excision, with the exceptions noted below. The 1 h and 5 h livers were frozen for periods longer than one week, then thawed for measurements. In addition, some measurements of the 24 h livers were repeated after freezing and thawing, to check for changes caused by this procedure. No significant differences were noted in the 24 h livers measured fresh, then after undergoing a freeze–thaw cycle. Previous studies have also borne out the negligible effect of careful freezing and thawing on tissue attenuation [24]. Absorption measurements were obtained using 2 mil chromel-constantan thermocouples inserted approximately 1 mm deep in the main lobe of the liver. The liver was positioned so the thermojunction was

TABLE I
TEMPORAL RESPONSE OF RAT LIVERS TO $CC\ell_4$ POISONING; 0.25 mg $CC\ell_4$/100 g DOSE

TIME (HOURS)	BIOCHEMICAL CHANGES	CELLULAR AND STRUCTURAL CHANGES
0-1	- lipid peroxidation of endoplasmic reticulum with resulting depression of protein synthesis, depression of glucose-6-phosphotase, cytochrome P450, and other enzyme activity. - initiation of triglyceride accumulation. - intracellular calcium redistribution.	- some centrilobular cells show initial signs of disorganization of endoplasmic reticulum; initial swelling of mitochondria, and an increase in fat droplets.
1-5	- doubling of normal triglyceride levels. - doubling of mitochondria calcium content.	- swelling of mitochondria. - cell and organ swelling. - fat droplets appear in the cytoplasm and mitochondria.
5-24	- peak accumulation of liver neutral lipids (early on) followed by declining triglyceride levels. - continued increases in mitochondrial calcium content.	- non-functioning mitochondria. - necrosis in centrilobular regions. - "hydropic" cells composed of vacuoles appear in the midzonal regions. - cells in the peripheral regions may be unaffected or may contain large fat droplets.

located at the focal point of ultrasonic beams. Rate of heating [45] or thermal pulse-decay [46] measurements were taken in order to calculate absorption coefficients.

Attenuation measurements were obtained using the CW radiation force insertion loss technique [47], which has the advantages of being a phase insensitive method. To obtain adequate thickness of liver material, three to four livers were positioned in a 2 in diameter pillbox shaped container which incorporated Saran Wrap front and back parallel surfaces. The width of the sample holder was adjusted to carefully pack the livers into a nearly solid, parallel mass of thickness 0.8–1.2 cm. The preparations were performed under water in chilled, degassed saline, and visual inspection and pulse echo examination were used to verify the lack of fluid or bubble filled voids within the sample holder. The resulting attenuation coefficients are therefore true aggregate measurements over groups of livers. The excess attenuation caused by the thin Saran covers was measured independently and eliminated from the data presented herein.

Backscatter estimates were obtained semiquantitatively by observing the average peak-to-peak magnitude of the backscattered signal within the first half centimeter of the main lobe of livers, and comparing the signal amplitude with those obtained from a perpendicular specular reflector (aluminum block) and silicone absorber of known reflectivity, positioned at the same range (5 cm). A 10 MHz, 1/2 in diameter, unfocused wide-band ceramic transducer was used with a Panmetrics pulse-echo transmit receiver. Average backscattered echos were read from an oscilloscope trace, neglecting front and back lobe echos and rarely occurring large vessel echos.

All intensity levels and path lengths were sufficiently small as to avoid nonlinear, finite amplitude effects, as verified by separate measurements with a PVDF hydrophone. The accuracy of the attenuation, absorption and backscatter measurements are estimated to be ± 7 percent, ± 15 percent, and ± 3 dB, respectively, based on equipment limitations and variations on measurements of reference samples. The sample-to-sample variations in any liver group were also within the above limits.

RESULTS

Attenuation values were found to be time varying, peaking at 5 h postinjection, and dropping below normal levels at 24 h. The lowered attenuation was most pronounced near 1 MHz, as shown in Fig. 1. Data are presented as α/f versus f in order to emphasize the frequency dependence of attenuation. If the attenuation increased linearly with attenuation, then the data so presented would lie on horizontal lines. As can be seen in Fig. 1, the attenuation coefficients obtained for normal livers and all $CC\ell_4$ injected livers have a positive slope, indicating attenuation increases faster than frequency to the first power. Accordingly, curve fitting of the data were performed, and the results are given in Table II. The attenuation of normal livers, and the 1 h and 5 h samples had a similar power law dependence of frequency to the 1.10, 1.13, and 1.12 power. The 24 h liver, in comparison, fits a power law of frequency to the 1.25 power, but with a marked decrease in the overall magnitude. This combination of decreased magnitudes coupled to increased power law coefficients has been noted in clinical studies [6], [48].

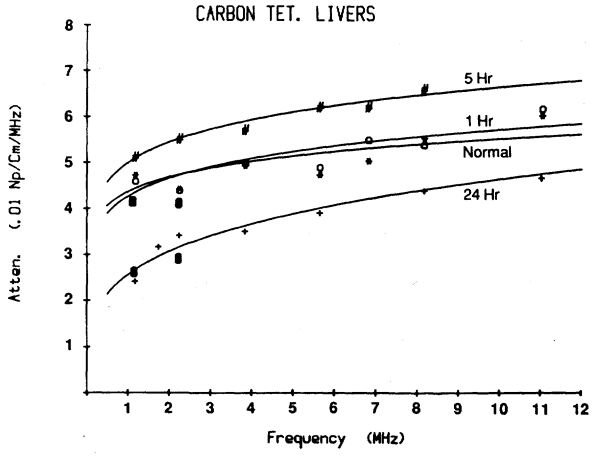


Fig. 1. Attenuation divided by frequency (np/cm · MHz) versus frequency (MHz) for normal livers and those excised 1, 5, and 24 h after injection of $CC\ell_4$. Solid lines are power law curve fits of the data using parameters given in Table II. Absorption values (solid dots) were obtained at 1 and 2 MHz in normal and 24 h livers.

TABLE II
CURVE FIT OF ATTENUATION TO A POWER LAW: $\alpha(f) = \alpha_0 f^n$

	α_o np/cm/MHz^n	n
NORMAL	0.044	1.10
1 HR.	0.043	1.13
5 HR.	0.051	1.12
24 HR.	0.026	1.25

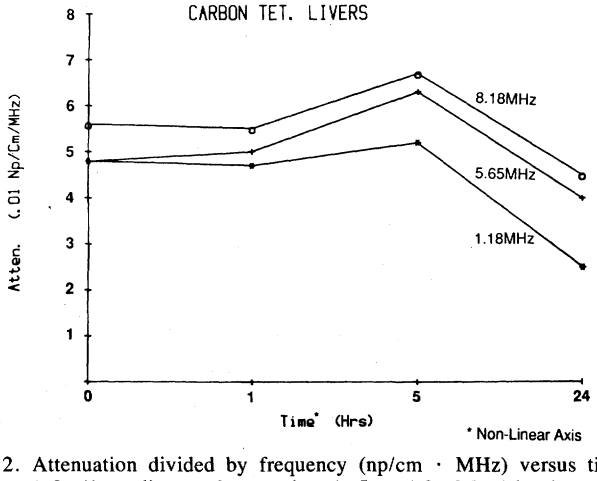


Fig. 2. Attenuation divided by frequency (np/cm · MHz) versus time (hours) for three discrete frequencies; 1, 5, and 8 MHz. The time axis in nonlinear.

Measured values of attenuation at three discrete frequencies are presented as a function of time in Fig. 2. The changes are frequency dependent, with marked increases in attenuation at 5 h for both 5 and 8 MHz measurements, but not 1 MHz. Also, the drop in values at 24 h in most pronounced at 1 MHz.

Absorption measurements obtained at 1.18 and 2.25 MHz closely match the attenuation data at these frequencies. At higher frequencies, focused beamwidths were smaller than 3 mm, and under these conditions heat conduction effects preclude rate-of-heating measurements.

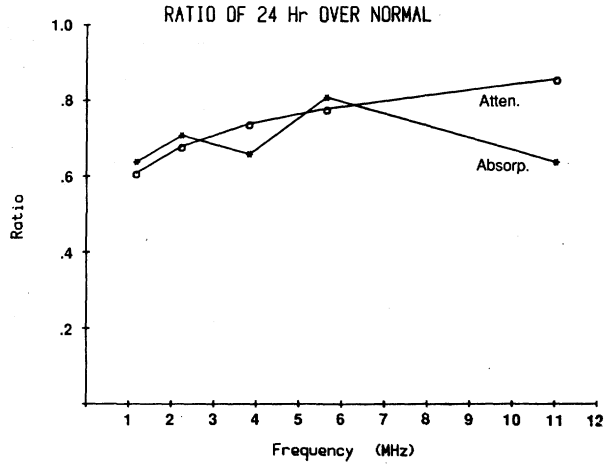


Fig. 3. Ratio of "24 h" values to "normal" values of both attenuation and absorption. Both absorption and attenuation are seen to drop to 60–80 percent of normal levels at 24 h postinjection.

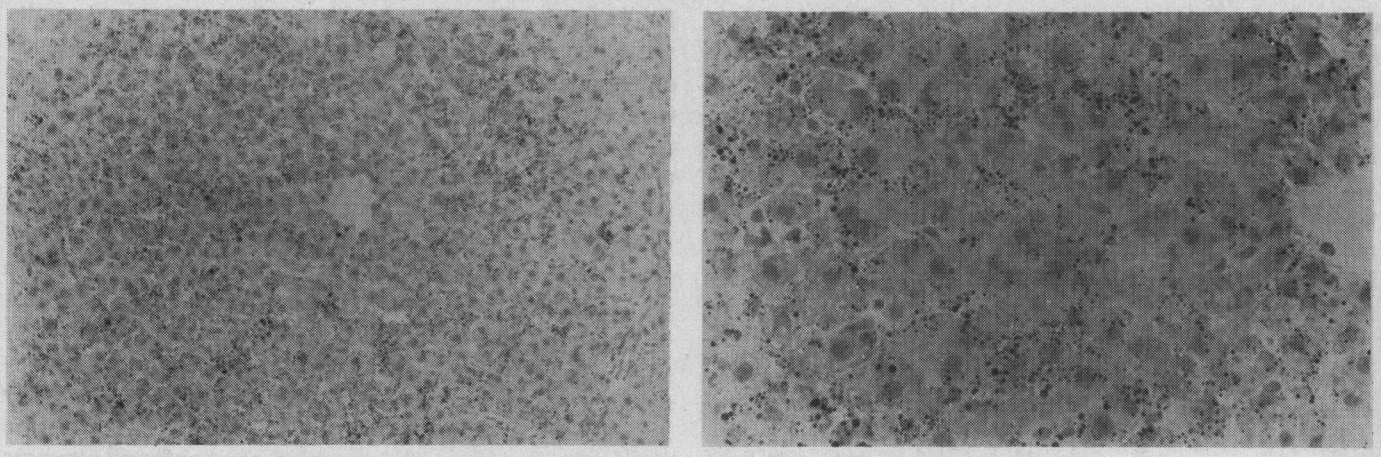
The focal patterns used at higher frequencies (3.84, 5.65, and 11.05 MHz) were also non-Gaussian, so application of pulse decay theory was not justifiable. In these cases, the absolute value of absorption coefficients could not be obtained. However, a comparison of rate of heating measurements taken on the normal and 24 h livers could be obtained, as the correction factor caused by beamwidth and conduction effects would be the same in both cases. It was found that the ratio of absorption values from the 24 h group to absorption values of normals was close to the ratio of total attenuation in the 24 h group compared to attenuation of the normals. The results are shown in Fig. 3. Thus, the lowering of absorption and attenuation values at 24 h are quantitatively similar.

Average backscatter echo amplitudes were found to be between −57 and −61 dB below the echo amplitude from a perfect reflector, irrespective of the liver history. Because of the small sample sizes and close grouping of results, no significant differences in backscatter coefficients of liver, before and after $CC\ell_4$ toxicity, can be inferred.

Gross changes in tissue structure were verified by histological examination of 24 h livers. Oil Red O. stain was used to emphasize fat droplets. Figs. 4 and 5 shows representative sections of both normal and 24 h livers. In the fatty liver, distinct regions of necrosis, accumulation of high density of fat droplets, and some unaffected areas can be seen. The fat droplet diameters in the $CC\ell_4$ damaged livers lie principally within the 1–5 μm range, and appear in Fig. 5 as dark grey-black droplets against the lighter grey background of cells.

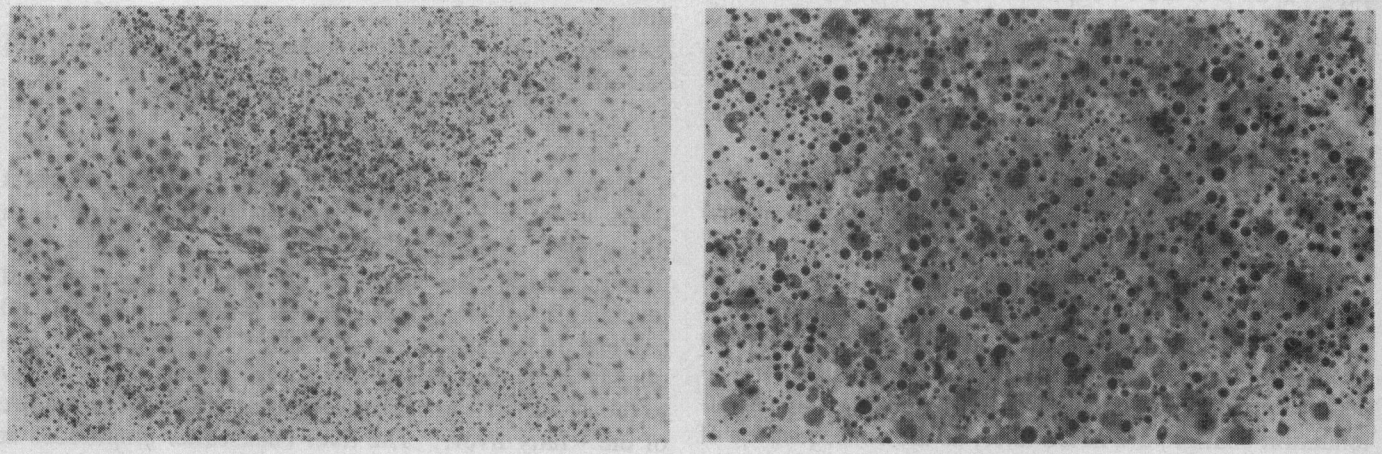
DISCUSSION

There appears to be no simple relationship between any single component of the many biochemical and structural changes in the rat liver resulting from $CC\ell_4$ toxicity (Table I) and the corresponding change (or lack of change) in ultrasonic absorption, attenuation, and backscatter coefficients. For example, a simple hypothesis might center on the increase in triglycerides which is underscored by the phrase "fatty liver." Since fat is often reported to



(a) (b)

Fig. 4. Normal liver sections at 40× (a) and 100× (b). Nuclei are stained to neutral gray, and fat droplets to dark grey-black.



(a) (b)

Fig. 5. Sections of a rat liver excised 24 h postinjection, at 40× (a) and 100× (b). The fat droplets droplets appear as dark grey-black, and are seen to increase in average size and concentration, compared to normal livers. Droplet sizes range from below 1 μm to approximately 5 μm.

have lower attenuation than normal liver [40], [43], the increase in fat content, coupled with cellular and whole organ swelling (presumably with low attenuating, plasma-like fluid), would act to lower the attenuation as a function of time. Also the dramatic increase in the size and number of fat droplets, presumably having different density and compressibility from the surrounding intra- and extracellular components, might be expected to change the backscatter coefficient, since incoherent Rayleigh backscatter would increase with the particle concentration, and the radius to the sixth power.

The above hypothesis does not adequately explain the observed changes, however. First, the increase in attenuation at 5 h postinjection is not accounted for, even though the accumulation of liver neutral lipids can peak at well over twice the normal levels in this time period. Second, although the attenuation is lowered at 24 h postinjection, the frequency dependence of attenuation no longer has the same form as the "normal" curve. This would not be expected, given a simple dilution of normal liver with saline and triglycerides. Furthermore, there appears to be an unusual drop in attenuation below 1.5 MHz. While it is tempting to discount the attenuation value in Fig. 1 for the 24 h livers at 1.18 MHz, we emphasize that these measurements in particular were repeated after freezing and thawing, and the measurements proved repeatable. Although the drop in attenuation values for the 24 h livers appears precipitous between 1 and 2 MHz, a log–log plot of these low frequency data show that attenuation increases as frequency to the 1.4 power within this octave. Thus, a single power law fit for the 24 h data is not adequate to describe the data. Additional measurements of attenuation around 0.5 MHz would be desirable, but could not be obtained in this study due to the need for larger sample thickness caused by very low attenuation values.

Another problem with the simple hypothesis stated above is that the expected changes in backscatter coefficients caused by the fat droplets were not observed at least within 3 dB limits of uncertainty in the measurements and variations in the liver samples themselves. The lack of dramatic change in backscatter amplitude is interesting

when one considers that, in the fatty liver, inhomogeneities are enhanced not only on the micron scale (resulting from fat droplets), but also on the millimeter scale, as necrosis tends to be localized in the central regions of the lobules. (See Table I and Fig. 4.) It is possible that the swept angle, swept frequency scattering measurements of Campbell and Waag [18] would detect these changes, as more forward scattering would be expected from lobular inhomogeneities having a characteristic length of nearly a millimeter.

An alternative explanation for the time-varying attenuation values would be to assume that increased triglycerides cause a higher than normal attenuation. Some evidence for this was given recently by Tervola *et al.* [39], although the roles of other factors, particularly collagen, were not determined. If we assume increased triglycerides at 5 h cause increased attenuation, then the drop in attenuation at 24 h could be explained by the subsequent tissue swelling and necrosis. One problem with this hypothesis is that the 5–24 h attenuation changes cannot be explained by a simple dilution effect, since changes in the attenuation frequency dependence are observed, along with the decrease in magnitude.

It is interesting to note that attenuation coefficients in 1 h postinjection livers are close to the values measured from normal rat livers. In some respects this is surprising, since in the first hour the damage sequence is well underway. Table I shows in particular that disruption of the endoplasmic reticulum of the hepatocytes, and subsequent changes in many important biochemical pathways, are developed in the first hour. However, more significant attenuation changes are noted later, as the consequences of these initial disruptions are manifested.

The closeness of attenuation and absorption coefficients warrants additional comments. In both the normal and 24 h livers, it appears that absorption is the dominant component of total attenuation at 1.18 and 2.25 MHz. This reinforces the conclusions of previous studies on bovine liver [18], [23], and implies that scattering does not contribute significantly to total attenuation in either normal or damaged rat livers. While absolute absorption coefficients could not be calculated at higher frequencies, the ratio of absorption of 24 h livers to absorption of normal livers is included in Fig. 3. The close fit of the absorption ratio to the attenuation ratio over all frequencies, combined with the known match of absorption and attenuation values at 1.18 and 2.25 MHz, provides evidence that absorption continues to be the dominant component of total attenuation up to 11 MHz, in both the normal and fatty livers.

These results are relevant to clinical studies of attenuation *in vivo*. First, the data presented herein show that attenuation coefficients can be time varying. Thus, a characterization of this form of "fatty liver" could not be given by a simple higher-than-normal or lower-than-normal generalization without regard to the staging of the "disease," or time dependence of the measurements. This may prove to be the case in forms of cirrhosis in humans, for example. Equally important, the attenuation results show that both the magnitude and frequency dependence of attenuation are important parameters. Some measurements of "attenuation" are in fact estimates of an attenuation "slope," [13] which is related simply to true attenuation only in cases where it increases linearly with frequency. As shown by Fig. 1, the attenuation in normal rat livers may increase linearly with frequency over the low MHz band; however, the abnormal livers have a more complex relationship between attenuation and frequency. It has been shown [16] that under these conditions the attenuation "slope" estimates can yield significant errors. Thus, a wide scatter in early *in vivo* liver attenuation measurements may be expected because the time-varying nature of attenuation can confound attempts to loosely categorize an individual disease or condition, and measurements of attenuation "slope" can yield erroneous results where attenuation does not bear a simple linear relation to frequency. The measurement of true attenuation magnitude and frequency dependency [5], [6] can eliminate the latter problem.

Conclusion

Ultrasonic attenuation and absorption coefficients of rat livers undergo significant changes within the first 24 h following injection of a sublethal dose of $CC\ell_4$. Attenuation increases approximately 20 percent above normal levels at 5 h, then drops to levels which are only 60–80 percent of normal levels at 24 h. The changes are frequency dependent and do not appear to be related simply to the accumulation of triglycerides, which is one feature of the "fatty liver." The liver backscatter amplitudes at 10 MHz do not appear to vary significantly with time. Scattering is shown to be a minor contributor to total attenuation in the normal and damaged rat livers, where absorption is the dominant loss mechanism. The present study provides two conclusions regarding clinical studies of attenuation; tissue coefficients may be time varying, and the assumption of linear increase of attenuation with frequency cannot be relied on as an *a priori* basis for calculations.

Further research may lead to a better understanding of the relationships of attenuation, absorption, and scattering to tissue components, in normal and diseased states.

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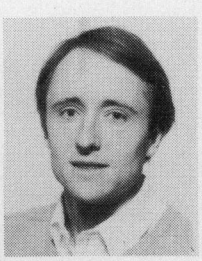

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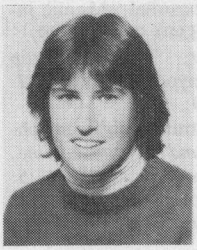

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