Ultrasonic propagation properties (@ 100 MHz) in excessively fatty rat liver

William D. O'Brien, Jr.
Bioacoustics Research Laboratory, Department of Electrical and Computer Engineering, University of Illinois, 1406 W. Green Street, Urbana, Illinois 61801

John W. Erdman, Jr.
Department of Food Science, University of Illinois, 905 S. Goodwin, Urbana, Illinois 61801

Tammy B. Hebner
Bioacoustics Research Laboratory, Department of Electrical and Computer Engineering, University of Illinois, 1406 W. Green Street, Urbana, Illinois 61801

(Received 15 July 1987; accepted for publication 23 November 1987)

The effects on the ultrasonic propagation properties of livers of the addition of 1% orotic acid to rat diets were examined. In rats, dietary orotic acid exerts several effects on lipid metabolism; its overall consequence is that excessively high hepatic fat concentrations are built up over a short period of time, thus making this an ideal model to study the ultrasonic propagation properties as a function of sequential development of fatty liver. Over a 16-day period on the orotic acid diet, the supplemented rat liver lipid concentrations increased from a normal range of 2%–4% to the lower 20's%; hepatic water concentration decreased from a normal value of approximately 70% to approximately 50%; total protein concentration decreased slightly from a normal range of 17%–20% to 11%–16%; and rat liver weight increased from approximately 11 g to around 20 g. Ultrasonic attenuation coefficient and speed were assessed in liver tissue with the scanning laser acoustic microscope at 100 MHz. As hepatic lipid increased, ultrasonic attenuation at 100 MHz increased temporally from a normal range of 12–14 dB/mm to a maximum of 54 dB/mm and ultrasonic speed decreased from a normal range of 1553–1584 m/s to a minimum of 1507 m/s. Multivariant linear regression was used in the analysis of covariance to fit the least-squares estimates to the linear regression model. Strong correlates of ultrasonic speed with both water concentration and fat concentration in the liver were observed.

PACS numbers: 43.80.Cs, 43.80.Ev, 43.80.Jz

INTRODUCTION

Considerable insight has been gained into the mechanisms responsible for the interaction of ultrasound in aqueous solutions of biomolecules through ultrasonic spectroscopy, that is, the examination of these media as a function of state variables such as frequency and temperature and as a function of media parameters such as concentration.[1,2] The same approach is now being applied to understand how ultrasonic energy interacts with intact tissue.

It has been known for three decades that the ultrasonic propagation properties, particularly the attenuation coefficient, of biological materials are strongly affected at the macromolecular level.[3–5] Four tissue constituents that are of particular importance acoustically are water, protein, collagen, and fat. A comparison of ultrasonic absorption, attenuation, and speed to the concentrations of these tissue constituents has suggested that the ultrasonic propagation properties of tissue can be modeled as functions of the constituent concentrations.[6,7]

Fat has long been thought to possess an ultrasonic attenuation that might be slightly lower but not unlike that of many of the parenchymal tissues. Therefore, fat concentration has not received serious consideration to be an important tissue constituent for characterizing tissues using ultrasound. However, fat does have an ultrasonic speed some 50–100 m/s less than most other soft tissues, and there is evidence to suggest that in subcutaneous fat, in particular, speed is as much as 300–600 m/s lower.[8] For these reasons, we have begun to consider fat concentration as a possible tissue characterizing quantity. Ultrasonic attenuation coefficient and speed have previously been determined with the scanning laser acoustic microscope (SLAM) in rat liver as a function of dietary ethanol-induced fat changes (fat content varied from 2.5% to 16.8%).[9] The results showed that the attenuation coefficient increased and the speed decreased as the fat content increased. Also, the attenuation coefficient of nearly pure fat tissue (near rat abdominal wall) appeared to have a value nearly four times greater than that of normal liver tissue at 100 MHz, about 50 dB/mm.

The current study was conducted to evaluate the ultrasonic propagation properties in liver in which the fat concentration increased excessively beyond normal limits, that is, up to and exceeding 20%. It has been known for some time that dietary orotic acid causes liver lipid accumulation in rats.[10,11] The orotic acid-induced fatty liver is apparently unique to rats as the response has not been seen in other species examined including mice, hamsters, Guinea pigs,

 0001-4966/88/031159-08$00.80

chicks, rabbits, dogs, pigs, and monkeys.[11] Thus the orotic acid-fed rat is an ideal model for studying the ultrasonic propagation properties as a function of fat accumulation in liver. For completeness, total protein, hydroxyproline, and water were also quantified and evaluated in terms of their influence on the ultrasonic propagation properties at 100 MHz.

I. METHODS

A. Experimental protocol

Twenty-four female, ex-breeder, Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), ranging in weight from 246 to 350 g, were fed lab blocks (Purina) for 5 days prior to commencing the study. Sixteen of the rats were then placed on a 1% dietary orotic acid semipurified diet (see Table I for diet). The other eight rats were fed the same semi-purified diet without orotic acid. In the orotic acid diet, 1% of the starch was replaced by orotic acid. All animals were kept individually in wire-bottomed cages in a room controlled for temperature (20–22 °C) and light (from 8:00 a.m. to 8:00 p.m.). Water and diet were both available *ad libitum*. Two orotic acid-fed rats and one control-fed rat were killed on days 5, 6, 7, 8, 13, 14, 15, and 16 after feeding, day 0 being defined as the time when the experimental diets commenced.

On each kill day, three rats were anesthetized with an overdose of ether. The livers were quickly removed, weighed, and prepared for both biochemical and ultrasonic analyses. Approximately one-half of the tissue was immediately frozen for biochemical analysis and the balance was placed in normal saline at room temperature for ultrasonic analysis.

B. Ultrasonic procedures

The ultrasonic attenuation coefficient and speed measurement techniques have been reported in detail[12,13] and have been previously used by this laboratory to study frozen, then thawed liver tissue.[9] All ultrasonic measurements in the current study were made at room temperature (22°) within 3 h from the time the rat was killed.

TABLE I. Diet composition. Casein, mineral mix, and vitamin mix supplied by Teklad Test Diets (Madison, WI), choline chloride by ICN Pharmaceuticals (Cleveland, OH), and anhydrous orotic acid (#0-2750) and DL methionine by Sigma (St. Louis, MO).

Ingredients	1.0% Orotic acid (g/100 g)	Control (g/100 g)
casein (vitamin free)	20.00	20.00
DL methionine	0.15	0.15
AIN 76 mineral mix	3.50	3.50
AIN 76 vitamin mix	1.00	1.00
choline chloride	0.20	0.20
corn oil	5.00	5.00
corn starch	45.77	46.77
sucrose	23.38	23.38
orotic acid	1.00	0.00
	100.00	100.00

The ultrasonic attenuation coefficient measurement technique[12] utilized the insertion loss method, which involved the comparison of the received signal amplitude with and without specimen of known thickness in the sound path. Four values of insertion loss were recorded for each of four thicknesses from each sample. The slope of the insertion loss versus thickness curve was determined by a least-squares analysis to yield the attenuation coefficient at 100 MHz.

The speed measurement technique[13] utilized the SLAM's interferometric mode, which provides the relative phase change of the wave after it has propagated through the specimen. The spatial frequency domain technique for determining ultrasonic speed yields approximately 35 values of the specimen under examination on the microscope. A single specimen's recorded speed value at a single thickness is the mean of these 35 values. These four mean speed values from the four thicknesses are then averaged to yield the speed of the specimen.

Based upon a separate study, the SLAM's measurement uncertainty was assessed with solutions of known acoustic properties.[14] This analysis yielded an accuracy of $\pm$ 12% and a precision of $\pm$ 15% for the attenuation coefficient and $\pm$ 2.9% and $\pm$ 0.4% for speed, respectively.

C. Biochemistry procedures

To determine total liver lipid,[15] liver samples (2–10 g) were homogenized with deionized water in a Polytron homogenizer (Brinkmann Instruments, NY) for approximately one minute. Generally 8–10 g were pooled for analysis, thus assuring a representative sample of liver. Two gram samples were utilized in only a few of the early control liver samples, which were uniform and low in fat. The polytron was rinsed with deionized water and a total volume was obtained to calculate a g/ml ratio of the homogenate. The polytron was rinsed with chloroform:methanol (2:1, by volume) between samples. Samples were analyzed in triplicate. A small volume of homogenate (0.3–1.0 ml) was vortexed with 15 ml of chloroform:methanol (2:1) and filtered through Whatman #1 filter paper. After the sample was allowed to filter, 3 ml of 0.29% NaCl was added and filtered. After stirring the two phases together, the sample was centrifuged for 20 min at 1500 rpm; the water layer was aspirated off and the sample was rinsed twice with 2–3 ml of 0.29% NaCl. The lipid layer was transferred to a tared test tube and the solvent dried off with a nitrogen evaporator (Organomation Assoc., Inc.) at 60 °C; the samples were left overnight in a vacuum dessicator; the weight of the lipids was determined gravimetrically. The percent lipid was calculated as follows:

$$\text{lipid (\%)} = \frac{\text{wt. of lipid (g)}}{\begin{pmatrix}\text{ml homogenate}\\ \text{analyzed}\end{pmatrix}\begin{pmatrix}\text{g/ml}\\ \text{ratio}\end{pmatrix}} \times 100. \quad (1)$$

For total protein determination,[16] blanks, standards, and samples were analyzed in duplicate. Bovine serum albumin containing 10 mg/ml served as a standard. Blanks contained 1.0 ml of deionized water. Due to the high protein content of the homogenates, the samples were diluted 1:10 with deionized water before assaying. To each tube containing varying amounts of standard and sample, the appropri-

ate amount of deionized water was added. Four ml of biuret reagent was then added to each tube. The contents of the tubes were mixed with a Vortex mixer and allowed to stand at room temperature for 30 min. Absorbance was measured at 540 nm with a Beckman 250 spectrophotometer. Total protein was calculated as follows:

$$\text{mg protein} = 20.181(\text{absorbance at 540 nm}) - 1.14 \quad (2)$$

and

$$\frac{\text{mg protein}}{\text{g liver}} = (\text{mg protein}) \times (\text{dilution factor}) \times \frac{1}{\left(\begin{matrix}\text{ml homogenate} \\ \text{analyzed}\end{matrix}\right)} \times \frac{1}{\left(\begin{matrix}\text{g/ml} \\ \text{ratio}\end{matrix}\right)}. \quad (3)$$

Hydroxyproline was measured by the modified assay of Stegemann and Stadler.[17,18] Liver homogenates were hydrolyzed for 4 h at 120 °C under pressure (22 psi) in an autoclave at a ratio of 10 mg of tissue (wet weight) to 1 ml of 6 M HCl. The assay is based upon the standard addition technique. Following hydrolysis, the samples were dried in a vacuum dessicator and diluted with a phosphate buffer. Concentrations of 0, 1, 2, and 4 μg of hydroxyproline/ml buffer were added to each of four 2-ml aliquots of sample. A blank consisting of 3 ml of buffer was also prepared. One and a half ml of 0.05 M chloramine-T solution was added to each tube and allowed to react 20–25 min at room temperature. Then, 1.5 ml of freshly prepared aldehydeperchloric acid reagent was added to each tube and placed in a 60 °C water bath for 15 min. The samples were read at 550 nm with a Beckman 250 spectrophotometer within 3 h. A least-squares regression analysis was performed. The point at which the absorbance is zero is the concentration of hydroxyproline due to the tissue. Corrections were then calculated for dilutions and final concentration of mg hydroxyproline/100 g tissue was obtained. Collagen concentration was estimated by assuming collagen to be 12.5% hydroxyproline.[18]

A small portion (0.20–0.55 g) of the liver was used to determine water concentration gravimetrically. Samples were assayed in duplicate. Samples were weighed, blotted dry, minced, weighed, placed in a 90 °C oven, and dried for 24 h.

D. Statistical procedures

Treatment and control means were compared between ultrasonic and biochemical measurements using the Statistical Analysis System.[19] An analysis of covariance (ANCOVA) was used to compare sample variances. Linear regression was used in the ANCOVA procedure to fit the least-squares estimates to the linear regression model. The interaction between tissue constituents was evaluated from the Pearson correlation coefficient ρ. Comparisons were considered significant at the $p < 0.05$ level.

II. RESULTS AND DISCUSSION

Specimen selection site is a critical concern in biological tissue studies. Thus every effort was made to minimize sampling variability in the current study. The fatty liver induction by orotic acid appeared visually to be uniform throughout the liver. Photographic evidence (not shown) supported a uniform and complete "whitening" of the entire liver, suggesting equal effects across the liver. Samples for acoustical measurements were always taken from the same liver lobe and same location in the lobe. Acoustical sample tissue was repooled with the tissue to be used for lipid, protein, and hydroxyproline analyses. Water content was performed on fresh tissue. Personal communication with Dr. James L. Robinson (Department of Animal Sciences, University of Illinois), who has extensive experience with feeding orotic acid to a variety of animals, revealed that only when one feeds levels of orotic acid at 0.2% or lower does one see a "blotchy" liver, i.e., heterogeneous deposition of fat.

Table II lists the biochemical and ultrasonic properties of livers as a function of treatment time from both orotic acid-fed and control-fed rats. The female, ex-breeder rats consuming control diet all gained body weight during the feeding period. However, since animals of this age are essentially full-grown, their rate of growth was small and varied from rat to rat. Orotic acid-fed rats either gained less weight or in three cases lost a few grams of weight during the test period. The mean weight gain for control and orotic acid-fed rats during the experiment was 17.6 ± 9.1 g and 6.1 ± 6.9 g, respectively. Orotic acid-fed rats consumed less diet (15.1 ± 2.2 vs 16.7 ± 2.5 g/day) thus contributing to the difference in total body weight. The weight of the liver was approximately 41% greater in the orotic acid treatment group as compared to the controls.

Liver weight in the orotic acid-fed group was significantly greater (41%) than controls ($p < 0.05$). The mean $\pm$ the standard deviation liver weight in the orotic acid group was 17.1 ± 2.3 g whereas that in the control group was 12.1 ± 0.9 g. There was no apparent statistically significant increase in liver weight in the orotic acid treatment group as a function of time on the diet. The majority of the liver weight increase occurred between the time the animals were initially placed on the diet and day 5, after which the weight appeared to remain elevated compared to that of the controls.

A. Ultrasonic propagation properties

The ultrasonic speed was consistently lower in the orotic acid treatment group compared to the control. The mean $\pm$ standard deviation ultrasonic speed for the control livers was 1570 ± 10 m/s. The ultrasonic speed of the orotic acid treatment group showed a gradual decrease between days 5 and 8 and an apparent leveling off for days 13 through 16. Considering only treatment days 13 through 16, the ultrasonic speed of the orotic acid treatment group was 3% lower than that of the control group (1519 ± 10 m/s vs 1567 ± 12 m/s).

The ultrasonic speed for the control livers agrees quite well with most other studies.[20–24] The ultrasonic speed was measured with the SLAM at room temperature in fresh sheep and cat livers[20] and in fresh rat liver[21] with respective ultrasonic speeds of 1565, 1567, and 1577 m/s. These speed values are also in excellent agreement with measurements in

TABLE II. Ultrasonic propagation and biochemical properties of livers from control-fed and orotic acid-fed rats as a function of time on their respective diets.

Days on diet (DOD)	Animal wt. (g) gain	Liver wt. (g)	Liver ultrasonic speed (m/s)	Liver ultrasonic attenuation coefficient (dB/mm)	Based on % wet weight		
					% Lipid	% Protein	% H_2O
Treatment: Control-fed							
5	16.0	13.2	1563	12.0 ± 0.8	2.9 ± 0.2	21.7 ± 2.1	73.5 ± 1.4
6	21.9	12.4	1567	13.2 ± 1.2	3.5 ± 0.3	17.1 ± 2.8	76.3 ± 2.2
7	12.3	11.9	1578	11.9 ± 1.3	3.4 ± 0.7	17.5 ± 0.3	72.9 ± 0.7
8	13.8	10.9	1581	12.2 ± 0.9	3.0 ± 0.7	16.6 ± 0.3	75.0 ± 1.0
13	20.6	13.0	1554	13.6 ± 0.8	2.5 ± 0.3	18.5 ± 0.9	71.3 ± 0.8
14	12.5	10.7	1567	13.3 ± 0.8	3.6 ± 0.4	21.0 ± 1.6	71.2 ± 0.0
15	36.6	12.4	1564	14.5 ± 0.7	4.8 ± 0.2	16.5 ± 1.7	69.2 ± 0.0
16	6.9	12.3	1584	13.5 ± 0.6	3.9 ± 0.3	21.3 ± 0.8	68.7 ± 0.3
Treatment: Orotic acid-fed							
5	1.0	16.8	1560	12.1 ± 0.7	13.5 ± 0.4	11.6 ± 0.4	65.1 ± 3.8
5	12.0	15.0	1549	15.4 ± 1.0	12.7 ± 0.4	12.5 ± 0.6	70.4 ± 1.0
6	6.3	15.5	1549	20.6 ± 0.9	14.8 ± 0.2	14.5 ± 0.6	62.6 ± 0.4
6	11.8	15.3	1538	22.9 ± 1.2	15.0 ± 1.3	15.8 ± 0.8	62.7 ± 1.9
7	− 6.7	13.2	1558	23.1 ± 1.8	15.8 ± 1.8	11.0 ± 0.2	65.6 ± 1.6
7	− 3.0	17.3	1525	20.0 ± 1.1	23.8 ± 1.0	11.6 ± 0.1	59.7 ± 4.7
8	0.2	19.6	1524	21.6 ± 0.9	26.5 ± 0.7	12.0 ± 0.6	54.5 ± 1.5
8	6.4	18.8	1531	22.5 ± 0.9	18.1 ± 0.7	18.4 ± 0.6	57.3 ± 2.2
13	9.4	21.2	1511	11.5 ± 1.0	19.8 ± 0.5	25.7 ± 1.2	50.5 ± 1.2
13	8.1	18.5	1509	10.3 ± 0.5	24.5 ± 0.3	19.3 ± 4.3	49.8 ± 0.4
14	15.0	13.4	1535	14.5 ± 0.5	19.6 ± 0.5	13.1 ± 0.3	59.4 ± 2.1
14	11.8	16.6	1520	14.6 ± 0.8	22.6 ± 0.4	11.1 ± 0.4	56.1 ± 3.4
15	17.8	20.0	1528	28.9 ± 2.2	21.1 ± 0.5	14.1 ± 3.3	54.5 ± 0.9
15	− 2.2	17.7	1513	28.1 ± 1.5	20.9 ± 0.8	19.6 ± 1.2	55.6 ± 4.7
16	6.1	16.4	1527	40.6 ± 5.8	23.9 ± 0.7	16.7 ± 1.4	53.4 ± 2.1
16	3.9	17.1	1508	53.8 ± 4.7	22.6 ± 1.5	16.7 ± 1.1	52.1 ± 0.7

the low MHz frequency range for beef liver where a value of 1566 m/s was reported[22] and for excised human liver at room temperature with values of 1570 m/s[23] and 1575 m/s.[24]

In a previous study in this laboratory,[9] slightly lower values of ultrasonic speed were determined in frozen, then thawed rat liver from male Sprague Dawley rats that were fed control and ethanol-containing diets for a period of 4 weeks. The ethanol diet produced a minor increase in the liver lipids (respective ranges of control and ethanol groups were 2.5%–3.3% and 3.2%–4.3% as compared to this study with female, ex-breeder, Sprague Dawley rats of 2.5%–4.8% and 12.7%–24.5%). The propagation speeds measured were 1550 and 1553 m/s. Total protein and water were not assessed in that study.

The ultrasonic attenuation at a frequency of 100 MHz was, in general, greater in the orotic acid treatment group as compared to the controls (exceptions on days 13 and 14). The mean ± standard deviation ultrasonic attenuation coefficient for the controls was 13.0 ± 0.9 dB/mm. This is approximately 28% less than that reported for fresh bovine liver (17.7 ± 2.2 dB/mm),[25] and quite similar to the ultrasonic attenuation coefficient determined in our laboratory for male Sprague Dawley rat liver in animals that were fed control and ethanol-containing diets for 4 weeks (13.0 and 15.6 dB/mm, respectively).[9]

Ultrasonic speed decreased and attenuation coefficient gradually increased due to orotic acid treatment as compared to the controls from days 5 through 8. During the time period from when the animals were initially placed on the orotic acid diet to day 5 there was a very slight decrease in the speed from that of the control and essentially no difference in the ultrasonic attenuation coefficient from that of the control. On day 13, the values for both ultrasonic quantities for the orotic acid treatment groups appeared to decrease from that of day 8. Speed decreased from 1527 to 1510 m/s and attenuation coefficient decreased from 22 to 11 dB/mm. For the attenuation coefficient, the orotic acid and control treatment groups were essentially the same for both days 13 and 14, after which the attenuation coefficient rose rapidly.

B. Biochemical properties

The liver's biochemical properties of lipid, protein, and water showed marked variations in the orotic acid treatment groups as compared to that of the controls. The mean ± standard deviation values (on a percentage of wet weight basis) for the controls for lipids, total proteins, collagen and water were 3.5 ± 0.7, 18.8 ± 2.2, 0.13 ± 0.093, and 72.3 ± 2.7, respectively. For control rats, these values remained relatively constant as a function of days on diet. In contrast, for the orotic acid-fed rats, the lipid concentration increased to over 20%, the protein concentration increased

slightly to almost 20%, and the water concentration decreased to around 50%.

There was a dramatic change in these biochemical properties during the first 5 days on the orotic acid treatment diet as compared to the controls. The lipid concentration increased by a factor of almost 4, the protein concentration decreased by approximately 9% before it started to increase, and the water concentration decreased approximately 3%.

Liver lipid increased significantly ($p < 0.05$) in the orotic acid group over controls. The mean liver lipid concentration of orotic acid-fed rats was 19.7% as compared to 3.5% for controls. The mean liver protein in the orotic acid-fed rats did not differ significantly from controls (15.2% vs 18.8%), but a slight decrease was observed. The mean water concentration was significantly lower ($p < 0.05$) in the orotic acid-fed group over controls (58.1% vs 72.3%).

There were no significant differences found between the orotic acid-fed and control-fed rats for liver collagen concentration. The mean $\pm$ standard deviation values for the control-fed rats for collagen concentration (g collagen/100 g of tissue) was 0.13 ± 0.093 (range 0.024–0.31). The mean collagen concentrations for the orotic acid-fed rats for days 5–8 and 13–16 on the diet were 0.15 ± 0.098 (0.095–0.35), and 0.17 ± 0.078 (0.098–0.29), respectively. Since collagen concentration was small and since there was no change between experimental groups, collagen was not included in the subsequent analyses.

Even though collagen has been shown to correlate with ultrasonic attenuation coefficient in the low megahertz frequency range,[6,7,26,27] this has been so only over a wide range of tissue collagen concentrations. In the case where seven tissues with approximately the same collagen concentration (hydroxyproline concentration: 0.013%–0.053%) were examined,[28] no statistically significant observation was obtained between attenuation coefficient and collagen concentration. This does not discount the influence of collagen; rather, it suggests that when the collagen concentration range is tightly grouped, it is not possible to observe an effect upon attenuation. When the collagen concentration range is broader, say for the study of canine wound tissue where the collagen concentration ranged from 10% to 25% (wet weight basis), both the attenuation coefficient (at 100 MHz) and speed show a statistically significant positive correlation.[27]

For the livers of the 16 orotic acid-fed rats, there were statistically significant negative interactions between lipid and water concentrations ($\rho = -0.82$; $p < 0.0001$; slope $m = \Delta\text{lipid}/\Delta\text{water} = -0.82$) and between protein and water concentrations ($\rho = -0.61$; $p < 0.012$; $m = \Delta\text{protein}/\Delta\text{water} = -0.93$). There was no statistically significant interaction between these three tissue constituent in the eight control livers. When the livers from all 24 rats were considered, there was a statistically significant negative interaction only between lipid and water concentrations ($\rho = -0.93$; $p < 0.0001$ $m = \Delta\text{protein}/\Delta\text{water} = -0.90$). Others[24] have also shown a statistically significant negative interaction between lipid and water ($m = \Delta\text{lipid}/\Delta\text{water} = -0.61$) for human liver over comparable lipid and water concentration ranges. This supports the view that the fat accumulation displaces water and protein.

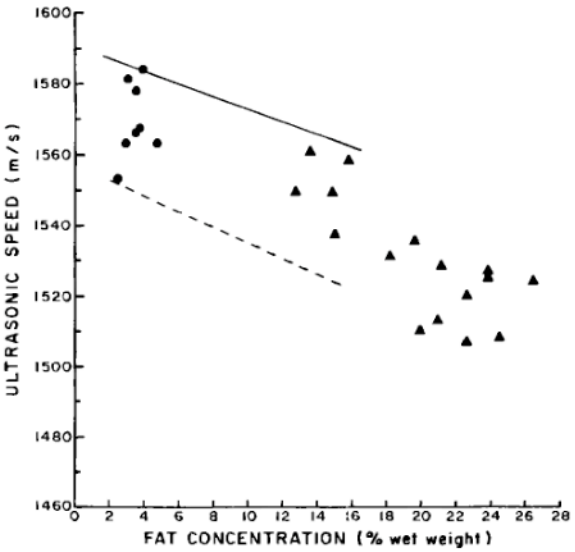

FIG. 1. Ultrasonic speed versus lipid concentration in rat livers for orotic acid (▲) and control (●) treatment animals. Dashed line from Ref. 9 and solid line from Ref. 24.

C. Comparison of ultrasonic and biochemical properties

The ultrasonic speed (m/s) and attenuation coefficient at a frequency of 100 MHz (dB/mm) were compared as a function of lipid, protein, and water (% wet weight basis) and are graphically represented in Figs. 1–6. The ultrasonic speed for the control livers is generally greater than that for the orotic acid treatment groups. Ultrasonic speed (Fig. 1) is shown to monotonically decrease as a function of increasing lipid concentration. Ultrasonic speed (Fig. 2) appears to

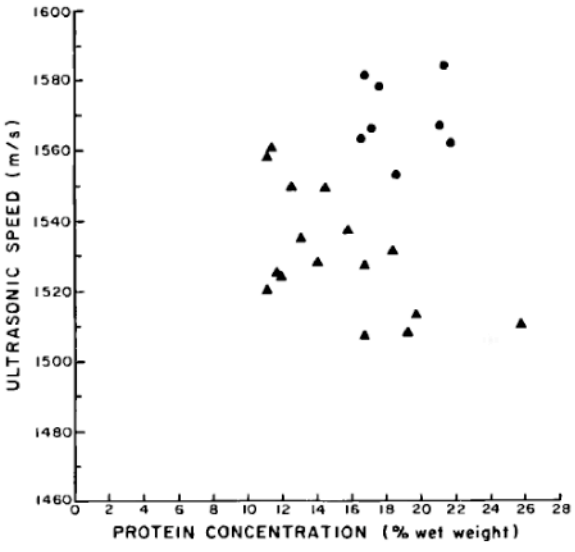

FIG. 2. Ultrasonic speed versus protein concentration in rat livers for orotic acid (▲) and control (●) treatment animals.

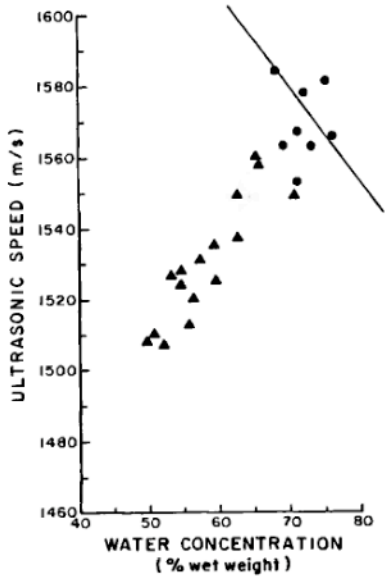

FIG. 3. Ultrasonic speed versus water concentration in rat livers for orotic acid (▲) and control (●) treatment animals. Solid line from Ref. 24.

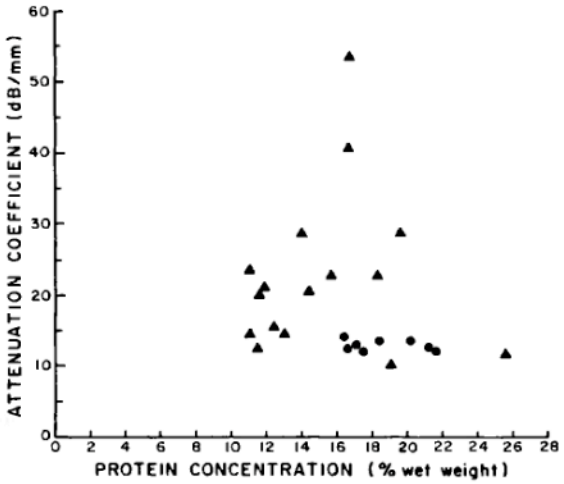

FIG. 5. Ultrasonic attenuation coefficient at 100 MHz versus protein concentration in rat livers for orotic acid (▲) and control (●) treatment animals.

decrease as a function of protein concentration for the orotic acid treatment group. The ultrasonic speed (Fig. 3) appears to monotonically increase as a function of increasing water concentration. The attenuation coefficient (Fig. 4) appears to remain the same or increase as a function of increasing fat concentration. There does not appear to be any obvious trends for the attenuation coefficients (Figs. 5 and 6) as a function of either protein or water concentration with the attenuation coefficient for the control livers either the same or less than that for the orotic acid treatment groups.

The dashed lines in Figs. 1 and 4 represent least-squares fit of ultrasonic speed and attenuation coefficient, respectively, versus fat concentration of livers from ethanol-fed rats over the fat concentration range (2.5%–16.8%) from a previous study.[9] Quantitatively in that study, speed was shown to decrease at a rate of 2.3 m/s/% fat and attenuation coefficient to increase at a rate of 1.08 dB/mm/% fat as functions of fat concentration. There appears to be quantitative agreement between these two studies. Further, others[24] have shown (solid line in Fig. 1) in normal human liver that the speed decreased at a rate of 1.8 m/s/% fat for the uncorrected data.

The ultrasonic speed versus water concentration results (Fig. 3) are distinctly different than reported by others.[24,27,29] The solid line in Fig. 3 represents human liver.[24] Here, the trend is opposite in slope although the human liver speed agrees in magnitude with that of the control rat livers in the 70% water concentration range. Others have also

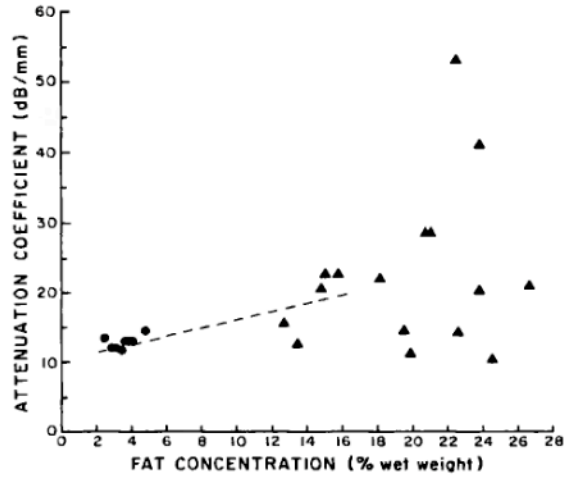

FIG. 4. Ultrasonic attenuation coefficient at 100 MHz versus lipid concentration in rat livers for orotic acid (▲) and control (●) treatment animals. Dashed line from Ref. 9.

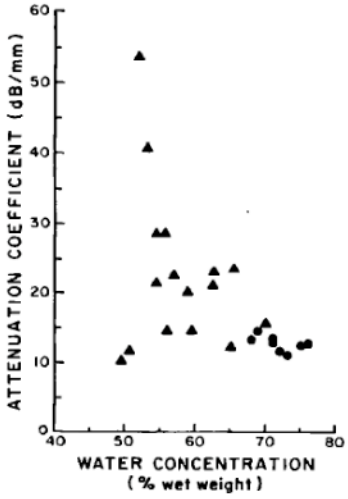

FIG. 6. Ultrasonic attenuation coefficient at 100 MHz versus water concentration in rat livers for orotic acid (▲) and control (●) treatment animals.

shown a negative slope in rabbit liver[29] and in canine wound tissue[27] over a similar water concentration range. No suggestion for this marked difference is known at this time.

Other studies[24,27] have shown statistically significant negative correlations between attenuation coefficient and water concentration for human liver in the 1–7 MHz range[24] and canine wound tissue at 100 MHz.[27] The results shown in Fig. 6 suggest a negative correlation, although very weakly.

These graphical representations, while useful, are also limited. They are able to depict a functional relationship between an ultrasonic quantity and one of the tissue quantities. However, it is felt that the interaction of ultrasound with biological materials is much more complex. Therefore, in order to obtain a more complete understanding of the functional relationships between the ultrasonic propagation properties and the tissue constituent properties, a more complete regression analysis was conducted in which each of the ultrasonic properties was evaluated in terms of all of the tissue properties and conversely, each of the tissue properties was examined against both of the ultrasonic properties.

Table III lists three sets of equations, each set representing regressions from (1) the 8 control-fed rats only, (2) the 16 orotic acid-fed rats only, and (3) all 24 rats. For each regression, an overall F statistic and probability level were determined, as well as a probability level for each independent variable in the equation. This approach was undertaken in order to assess the role of each of the independent tissue variables (lipid, protein, and water) on ultrasonic speed and attenuation coefficient.

Neither of the regressions from the control-fed rats was significant, most likely due to the fact that the data were tightly grouped which can be graphically observed in Figs. 1–6. Lipid and protein concentrations significantly influenced speed in the orotic acid-fed group whereas water did not. These two tissue constituents (lipid and protein) were not correlated according to the Pearson correlation coefficient. When the data from all 24 rats were combined, none of the independent variables significantly affected speed, based upon the $p<0.05$ criterion, although the overall probability level for the equation was significant.

Since the overall F value was less than 1.8 for each of the three attenuation coefficient equations in Table III, ultrasonic attenuation was not a significant estimator for lipid, protein, or water in the orotic acid-fed or control-fed treatment groups.

In addition, the biochemical measurements were used to estimate the ultrasonic propagation properties of speed and attenuation coefficient for the orotic acid-fed treatment group. Only this treatment group was selected for this analysis because of the apparent bias that is introduced by also including the control-fed treatment group (see Table III). The model fitted for lipid concentration is given by:

$$\begin{array}{llll} L = 317.5 & -0.2\ (c) & +0.02\ (A) & F = 10.8 \\ p<0.0005 & p<0.0008 & p<0.72 & p<0.002 \end{array} \quad (4)$$

The model for protein concentration is given by:

$$\begin{array}{llll} P = 248.3 & -0.2\ (c) & -0.04\ (A) & F = 4.0 \\ p<0.011 & p<0.015 & p<0.64 & p<0.045 \end{array} \quad (5)$$

The model for water concentration is given by:

$$\begin{array}{llll} W = -411.6 & +0.3\ (c) & -0.02\ (A) & F = 25.7 \\ p<0.0001 & p<0.0001 & p<0.78 & p<0.0001 \end{array} \quad (6)$$

It is evident for these regressions [Table III and Eqs. (4)–(6)] that little relationship existed between ultrasonic attenuation coefficient and any of the three tissue constituents considered in this study. On the other hand, speed was significantly influenced by lipid and protein concentration only. Further, from Eqs. (4)–(6), it was possible to make a significant estimate of all three tissue constituents from speed only. Tissue water concentration, even though correlated to tissue lipid and protein concentration, did not significantly influence ultrasonic propagation properties.

The increase in liver lipid due to orotic acid found in the present study was in agreement with others.[30,31] Liver lipids were shown to increase at 3 days and rise to around 18% after 7–10 days on a 1.0% orotic acid diet.[31] In the current study, liver lipids were first assayed after 5 days on the diet at which time they had increased from 2.5%–4.8% for control-fed rats to about 13%. Additionally, others[31,32] noted elevations to 25% lipid after 17 days on a 1.0% orotic acid diet. Still others[33] found no changes in rats fed 1.0% orotic acid until day 7 (10.2%) and further increases by day 10 (16.8%).

In this study, the length of time on the diet appeared to influence the lipid content in the orotic acid-fed animals. The large increase in liver lipid also resulted in a decrease in water concentration and a somewhat lower decrease in protein over controls. From these results, 1.0% orotic acid in the diet when fed to rats for a short period of time could drastically alter the lipid concentration and in doing so, affect the ultrasonic propagation properties and thus provided an excellent model for such studies.

TABLE III. Regression equations of the dependent variables speed (c, m/s) or attenuation coefficient (A, dB/mm) as a function of the three independent tissue variables, lipid (L), protein (P), and water (W), each as a percentage (wet weight basis). The overall F statistic and probability level for each equation is indicated to the far right. The p values listed below each coefficient represent the significance of that term to the dependent variable.

Treatment: Control-fed		
$c = 1455.4$	$+6.2(L)\ +0.7(P)\ +1.1(W)$	$F = 0.14$
$p<0.004$	$p<0.55\ \ p<0.80\ \ p<0.69$	$p<0.93$
$A = 29.3$	$+0.3(L)\ -0.1(P)\ +0.2(W)$	$F = 1.6$
$p<0.12$	$p<0.65\ \ p<0.49\ \ p<0.27$	$p<0.32$
Treatment: Orotic acid-fed		
$c = 1588.8$	$-2.5(L)\ -1.8(P)\ +0.3(W)$	$F = 24.6$
$p<0.0001$	$p<0.048\ \ p<0.05\ \ p<0.76$	$p<0.0001$
$A = 85.3$	$-0.2(L)\ -0.6(P)\ -0.9(W)$	$F = 0.43$
$p<0.59$	$p<0.93\ \ p<0.71\ \ p<0.62$	$p<0.73$
Treatment: Control-fed and ortic acid-fed combined		
$c = 1580.2$	$-2.4(L)\ -1.6(P)\ +0.4(W)$	$F = 36.2$
$p<0.0001$	$p<0.056\ \ p<0.084\ \ p<0.70$	$p<0.0001$
$A = 62.0$	$+0.1(L)\ -0.4(P)\ -0.6(W)$	$F = 1.8$
$p<0.52$	$p<0.93\ \ p<0.71\ \ p<0.58$	$p<0.18$

The decrease in ultrasonic speed and increase in ultrasonic attenuation with increasing lipid found in this study was consistent with previous work from this laboratory where mild fatty liver was induced in rats with chronic ethanol feeding.[9]

ACKNOWLEDGMENTS

Professor James L. Robinson, Department of Animal Sciences at the University of Illinois is gratefully acknowledged for suggesting the role of orotic acid in rats to study fatty livers. Daniel C. Colbert performed the SLAM measurements. This research was supported by the National Institutes of Health, National Cancer Institute (CA36029) and by the USDA-SEA competitive grant 5901-0410-9-0307.

[1]F. Dunn, P. D. Edmonds, and W. J. Fry, "Absorption and dispersion of ultrasound in biological media," in *Biomedical Engineering*, edited by H. Schwan (McGraw-Hill, New York, 1969), pp. 205–332

[2]F. Dunn and W. D. O'Brien, Jr., "Ultrasonic absorption and dispersion," in *Ultrasound: Its Application in Medicine and Biology*, edited by F. J. Fry (Elsevier, Amsterdam, 1978), pp. 393–439.

[3]E. L. Carstensen, K. Li, and H. P. Schwan, "Determination of the acoustic properties of blood and its components," J. Acoust. Soc. Am. **25**, 286–289 (1953).

[4]E. L. Carstensen and H. P. Schwan, "Acoustic properties of hemoglobin solutions," J. Acoust. Soc. Am. **31**, 305–309 (1959).

[5]H. Pauly and H. P. Schwan, "Mechanism of absorption of ultrasound in liver tissue," J. Acoust. Soc. Am. **50**, 692–699 (1971).

[6]W. D. O'Brien, Jr., "The relationship between collagen and ultrasonic attenuation and velocity in tissue," in *Ultrasonic International 1977* (IPC Science and Technology, Guilford, Surrey, UK, 1977), pp. 194–205.

[7]J. D. Pohlhammer and W. D. O'Brien, Jr., "The relationship between ultrasonic attenuation and speed in tissues and the constituents: Water, collagen, protein and fat," in *Medical Physics of CT and Ultrasound: Tissue Imaging and Characterization*, edited by G. D. Fullerton and J. A. Zagzebski (American Institute of Physics, New York, 1980), pp. 409–435.

[8]W. D. O'Brien, Jr., J. Olerud, K. K. Shung, and J. M. Reid, "Quantitative acoustical assessment of wound maturation with acoustic microscopy," J. Acoust. Soc. Am. **69**, 575–579 (1981).

[9]K. M. U. Tervola, M. A. Grummer, J. W. Erdman, Jr., and W. D. O'Brien, Jr., "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," J. Acoust. Soc. Am. **77**, 307–313 (1985).

[10]S. B. Standerfer and P. Handler, "Fatty liver induced by orotic acid feeding," Proc. Soc. Exp. Biol. Med. **90**, 270–271 (1955).

[11]K. K. Harden and J. L. Robinson, "Hypocholesteremia induced by orotic acid: Dietary effects and species specificity," J. Nutr. **114**, 411–421 (1984).

[12]K. M. U. Tervola, S. G. Foster, and W. D. O'Brien, Jr., "Attenuation coefficient measurement technique at 100 MHz with the scanning laser acoustic microscope," IEEE Trans. Sonics Ultrason. **SU-32**, 259–265 (1985).

[13]K. M. U. Tervola and W. D. O'Brien, Jr., "Spatial frequency domain technique: An approach for analyzing the scanning laser acoustic microscope interferogram images," IEEE Trans. Sonics Ultrason. **SU-32**, 544–554 (1985).

[14]D. L. Steiger, W. D. O'Brien, Jr., J. E. Olerud, M. A. Riederer-Henderson, and G. F. Odland, "Measurement uncertainty assessment of the scanning laser acoustic microscope and application to canine skin and wound," IEEE Trans. Ultrason. Ferroelec. Frequency Control (in press).

[15]J. M. Folch, K. Lees, and G. H. Sloane-Stanley, "A simple method for the isolation and purification of total lipids from animal tissues," J. Biol. Chem. **226**, 497–509 (1957).

[16]J. M. Clark, Jr. and R. L. Switzer, *Experimental Biochemistry* (Freeman, San Francisco, 1964), 2nd ed., p. 12.

[17]H. Stegemann and K. Stadler, "Determination of hydroxyproline," Clin. Chim. Acta **18**, 267 (1967).

[18]C. A. Edwards and W. D. O'Brien, Jr., "Modified assay for determination of hydroxyproline in a tissue hydrolzate," Clin. Chim. Acta **104**, 161–167 (1980).

[19]Statistical Analysis System Institute, Inc., *SAS User's Guide: Statistics, Version 5 Edition* (SAS Institute, Inc., Cary, NC, 1985), pp. 656–709.

[20]L. A. Frizzell and J. D. Gindorf, "Measurement of ultrasonic speed in several biological tissues," Ultrasound Med. Biol. **7**, 385–387 (1981).

[21]A. M. Mravca, "Semi-automated techniques to measure ultrasonic propagation properties using a scanning laser acoustic microscope," MS thesis, University of Illinois (1981).

[22]K. K. Shung and J. M. Reid, "Ultrasonic scattering from tissue," *1977 IEEE Ultrasonics Symposium Proceedings*, IEEE Cat. No. 77CH1264-SU, pp. 230–233 (1977).

[23]C. M. Sehgal, G. M. Brown, R. C. Bahn, and J. F. Greenleaf, "Measurement and use of acoustic nonlinearily and sound speed to estimate composition of excised livers," Ultrasound Med. Biol. **12**, 865–874 (1986).

[24]J. C. Bamber, C. R. Hill, and J. A. King, "Acoustic properties of normal and cancerous human liver-II. Dependence on tissue structure," Ultrasound Med. Biol. **7**, 135–144 (1981).

[25]J. D. Pohlhammer, C. A. Edwards, and W. D. O'Brien, Jr., "Phase insensitive ultrasonic attenuation coefficient determination of fresh bovine liver over an extended frequency range," Med. Phys. **8**, 692–694 (1981).

[26]R. L. Johnston, S. A. Goss, V. Maynard, J. K. Brady, L. A. Frizzell, W. D. O'Brien, Jr., and F. Dunn, "Elements of Tissue Characterization, Part I. Ultrasonic Propagation Properties," in *Ultrasonic Tissue Characterization II*, National Bureau of Standards, Special Publication 525 (U. S. Government Printing Office, Washington, DC, 1979), pp. 19–27.

[27]J. E. Olerud, W. D. O'Brien, Jr., M. A. Riederer Henderson, D. Steiger, F. K. Forster, C. Daly, D. J. Ketterer, and G. F. Odland, "Ultrasonic assessment of skin and wounds with the scanning laser acoustic microscope," J. Invest. Dermatol. **88**, 615–623 (1987).

[28]W. D. O'Brien and L. A. Segal, "Frequency dependent ultrasonic attenuation coefficient assessment in fresh tissue," Arch. Acoust. (Poland) **10**, 11–14 (1983).

[29]A. P. Sarvazyan, A. G. Lyrchilsov, and S. E. Gorelov, "Dependence of ultrasonic velocity in rabbit liver on water content and structure of the tissue," Ultrasonics (in press).

[30]R. E. Handschumacher, W. A. Creasey, J. J. Jaffe, C. A. Pasternak, and L. Hankin, "Biochemical and nutritional studies on the induction of fatty livers by dietary orotic acid," Proc. Natl. Acad. Sci. USA **46**, 178–186 (1960).

[31]W. A. Creasey, L. Hankin, and R. E. Handschumacher, "Fatty livers induced by orotic acid. I. Accumulation and metabolism of lipids," J. Biol. Chem. **236**, 2064–2070 (1961).

[32]H. G. Windmueller, "An orotic acid-induced, adenine-reversed inhibition of hepatic lipoprotein secretion in the rat," J. Biol. Chem. **239**, 530–537 (1964).

[33]R. P. Durschlag and J. L. Robinson, "Orotic acid-induced metabolic changes in the rat," J. Nutr. **110**, 816–821 (1980).