

## Effects of Intense Noncavitating Ultrasound on Selected Enzymes\*

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The denaturation of enzymes in solution by cavitating ultrasound has been reported previously. This report presents the results of an extensive study of the effects of noncavitating ultrasound on solutions of  $\alpha$ -chymotrypsin, trypsin, aldolase, lactate dehydrogenase, and ribonuclease. In one set of experiments, the solutions were irradiated and then analyzed to determine the effects on the physical and chemical properties of the enzyme molecules. Irradiations were carried out at different pH values and temperatures using 1-MHz ultrasound at an intensity of 75 W/cm<sup>2</sup>, 10-min continuous exposure, and 11-MHz ultrasound at an intensity of 1000 W/cm<sup>2</sup>, 2000 0.1-sec pulses. Analytical procedures employed included measurements of enzyme activity, specific optical rotation, uv absorption spectrum, and sedimentation coefficient. In a second set of experiments, enzyme-catalyzed reactions were irradiated with ultrasound and simultaneously monitored spectrophotometrically. Ultrasound in the intensity range 0.5–35 W/cm<sup>2</sup> at the frequencies 1, 9, and 27 MHz were employed with the temperature and pH held constant. Comparison of the results of this study with those from studies employing cavitation shows that cavitation is a necessary condition for ultrasonic denaturation of the five enzymes of this study.

### INTRODUCTION

IT is well known that microorganisms and cellular structures of higher organisms can be damaged extensively when subjected to ultrasonic cavitation.<sup>1</sup> It is less widely appreciated that selective alteration can be produced in tissue of the mammalian central nervous system by intense noncavitating ultrasound.<sup>2</sup> Developments in this latter field have provided an advantageous lesion-making method for neuroanatomical research,<sup>3</sup> and ultimately a versatile brain modifying means for therapy.<sup>4</sup> However, the need for a more adequate explanation of the physical mechanisms of interaction of intense noncavitating ultrasound and tissue structures remains. Experimentation with mammals has shown that physiological changes, e.g., limb paralysis,<sup>5</sup> resulting from intense ultrasonic irradiation of the spinal cord can be detected within seconds after exposure to a 1-sec pulse but that histological evidence of tissue alteration does not appear until approximately 10 min

after irradiation, with progressive lesion formation following.<sup>6</sup> It has been established, for such interactions, that cavitation,<sup>5</sup> the temperature increase occurring in the irradiated region of the specimen,<sup>5</sup> and unidirectional forces that might produce elastic failure of structural components when displacements from equilibrium positions occur,<sup>7</sup> are not responsible for the observed tissue changes. The temporary absence of detectable structural alterations in a functionally altered tissue suggested that the primary site of action of the ultrasound was a sub-microscopic structure, possibly a macromolecule. Reports that organic polymers,<sup>8</sup> the enzymes trypsin<sup>9</sup> and  $\alpha$ -amylase,<sup>10</sup> and DNA<sup>11</sup> could be degraded or denatured by exposure of solutions of these polymers to intense noncavitating ultrasound strengthened this suggestion.

In the study reported here, the enzymes  $\alpha$ -chymotrypsin, trypsin, aldolase, lactate dehydrogenase, and ribonuclease were selected for a detailed investigation of the interactions of noncavitating ultrasound with proteins in solution. The selection of enzymes was prompted by

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<sup>1</sup> I. W. El'piner, *Ultrasound: Physical, Chemical, and Biological Effects* (Consultants Bureau, Inc., New York, 1964), pp. 149–231.

<sup>2</sup> W. J. Fry, *Advan. Biol. Med. Phys.* 6, 281–348 (1958).

<sup>3</sup> W. J. Fry, F. J. Fry, R. Malek, and J. W. Pankau, *J. Acoust. Soc. Amer.* 36, 1795–1835 (1964).

<sup>4</sup> W. J. Fry and R. Meyers, *Confinia Neurol.* 22, 315–327 (1962).

<sup>5</sup> F. Dunn, *Amer. J. Phys. Med.* 37, 148–151 (1958).

<sup>6</sup> J. W. Barnard, W. J. Fry, F. J. Fry, and R. F. Krumins, *Comp. Neurol.* 103, 459–484 (1955).

<sup>7</sup> F. Dunn, *J. Acoust. Soc. Amer.* 29, 395–396 (1957).

<sup>8</sup> H. W. Melville and A. J. R. Murray, *Trans. Faraday Soc.* 46, 996–1009 (1950).

<sup>9</sup> V. Stefanovic, I. Kostic, M. Bresjanac, and D. Zivanovic, *Bull. Soc. Chim. Belgrade* 24, 175–178 (1959).

<sup>10</sup> V. Stefanovic, A. Djukanovic, K. Velasevic, and D. Zivanovic, *Experientia* 14, 486–487 (1960).

<sup>11</sup> S. A. Hawley, R. M. Macleod, and F. Dunn, *J. Acoust. Soc. Amer.* 35, 1285–1287 (1963).

several considerations. *First*, calculations based on acoustic absorption data for protein solutions<sup>12-14</sup> indicate that sufficient energy is available for the inactivation of enzyme molecules.<sup>15</sup> *Second*, previous investigations<sup>9,10</sup> indicated that enzymes were susceptible to inactivation by noncavitating ultrasound. *Third*, enzyme inactivation is a plausible explanation for the effects of intense noncavitating ultrasound on tissues of the mammalian central nervous system<sup>5</sup> since the vital activities of each cell depend upon the normal functioning of its enzymes. *Fourth*, it was felt that more could be learned by studying these relatively well-characterized proteins than any other class of biomacromolecules, e.g., their specific catalytic abilities are very sensitive to any alteration of structure that effects the "active site," and this provides a sensitive indicator for even very subtle changes in the nature of the enzyme's conformation. In addition, denaturation often is reliably reflected by changes in such physicochemical properties as sedimentation behavior, optical rotation, and the uv absorption spectrum. *Finally*, the selected enzymes were available in purified form, thus homogeneous samples of uniform size, shape, and molecular weight were studied rather than distributions of these characteristics.

Two types of studies were conducted. *First*, enzyme solutions were irradiated with intense noncavitating ultrasound and subsequently analyzed by various techniques (uv absorption spectra, optical rotation, sedimentation velocity analysis, chromatography) for changes in the physical and chemical properties of the enzymes. Temperature and pH of the solutions were varied in some cases to determine the effects of these variables on the interaction phenomena. *Second*, irradiations were carried out during enzyme-catalyzed reactions and these reactions were monitored continuously to determine whether reversible changes in enzyme structure occur that are completed too rapidly for detection by the above-mentioned techniques.

## I. MATERIALS AND METHODS

### A. Biochemical Materials and Methods of Analysis

All inorganic reagents were prepared from reagent-grade chemicals using distilled deionized water.

Trypsin (2X crystallized),  $\alpha$ -chymotrypsin (3X crystallized), aldolase (2X crystallized from rabbit muscle), and lactate dehydrogenase (2X crystallized from rabbit muscle) were obtained from the Worthington Biochemical Corporation. Bovine pancreatic ribonuclease (3X crystallized) was obtained from the Sigma Chemical Company. All the enzymes were used as obtained, without further purification. Fructose-1, 6-diphosphate and

sodium pyruvate were obtained from Mann Research Laboratories. Reduced nicotinamide-adenine dinucleotide (NADH<sub>2</sub>) was obtained from P-L Biochemicals, Inc., and cytidine 2':3' cyclic phosphate was obtained from Schwarz Bio Research, Inc. *N*-acetyl-L-tyrosine ethyl ester (ATEE) and *N*-benzoyl-L-arginine ethyl ester (BAEE) were obtained as "Determatubes" from the Worthington Biochemical Corporation.

Tris[tris(hydroxymethyl)aminomethane] buffer<sup>16</sup> and phosphate buffer<sup>17</sup> were prepared according to established procedures.

Trypsin,<sup>18</sup>  $\alpha$ -chymotrypsin,<sup>18</sup> aldolase,<sup>19</sup> lactate dehydrogenase,<sup>20</sup> and ribonuclease<sup>16</sup> activities were assayed spectrophotometrically with a Beckman DU spectrophotometer equipped with a thermostated (25.0° ± 0.1°C) cuvette compartment and a Sargent SRL recorder. Enzyme concentrations were determined spectrophotometrically, using published absorptivities for trypsin,<sup>21</sup>  $\alpha$ -chymotrypsin,<sup>21</sup> aldolase,<sup>22</sup> lactate dehydrogenase,<sup>23</sup> and ribonuclease.<sup>24</sup>

All pH measurements were made with a Beckman Zeromatic pH meter.

Ultraviolet absorption spectra, in the wavelength range 240-320 m $\mu$  were read either with the Beckman DU spectrophotometer used for activity assays (without the recorder) or with a Cary model 14 automatic scanning and recording spectrophotometer.

Optical rotation measurements were made with a Rudolph model 70 precision polarimeter using a 10.00-cm microtube (0.7-ml volume) and a filtered sodium-vapor light source.

Sedimentation velocity analyses were performed with a Spinco model E analytical ultracentrifuge equipped with schlieren optics and a RTIC unit. An An-D rotor was employed in all experiments. Both a standard cell with a Kel-F centerpiece and a valve-type synthetic boundary cell were used. The rotor speed setting for all experiments was 56 100 rpm. The photographic plates were measured with a comparator and sedimentation coefficients were determined in the usual way.<sup>25</sup> The sedimentation coefficients were converted to the standard conditions of water as solvent and 20°C.

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<sup>17</sup> G. Gomori, *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press Inc., New York, 1955), Vol. I, pp. 138-146.

<sup>18</sup> G. W. Schwert and Y. Takenaka, *Biochem. Biophys. Acta* **16**, 570-575 (1955).

<sup>19</sup> V. Jagannathan, K. Singh, and M. Damodaran, *Biochem. J.* **63**, 94-105 (1956).

<sup>20</sup> A. Kornberg, *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press Inc., New York, 1955), Vol. 1, pp. 441-443.

<sup>21</sup> B. C. W. Hummel, *Can. J. Biochem. Physiol.* **37**, 1393-1399 (1959).

<sup>22</sup> T. Baranowski and T. Niederland, *J. Biol. Chem.* **180**, 543-551 (1949).

<sup>23</sup> J. B. Neilands, *J. Biol. Chem.* **199**, 373-381 (1952).

<sup>24</sup> J. J. Hermans and H. A. Sheraga, *J. Amer. Chem. Soc.* **83**, 3283-3292 (1961).

<sup>25</sup> H. K. Schachman, *Methods in Enzymology* (Academic Press Inc., New York, 1957), Vol. IV, pp. 32-71.

<sup>12</sup> E. L. Carstensen and H. P. Schwan, *J. Acoust. Soc. Amer.* **31**, 305-311 (1959).

<sup>13</sup> P. D. Edmonds, *Biochim. Biophys. Acta* **63**, 216-219 (1962).

<sup>14</sup> S. A. Hawley, PhD thesis in biophysics, Univ. of Illinois, Urbana, Ill. (1967).

<sup>15</sup> R. M. Macleod, PhD thesis in biophysics, Univ. of Illinois, Urbana, Ill. (1966).

Thin-layer chromatography experiments were performed using the Eastman Chromagram Developing apparatus and Eastman Chromagram Sheets, type K301R. The solvent system used was *n*-butanol:acetic acid:water (3:1:1). Spots were located first by examining under uv light and then by spraying with a solution of ninhydrin in absolute ethanol (0.3g/100ml), and heating at 90°C for 15 min.

### B. Ultrasonic Instrumentation: Irradiation of Enzyme Solutions

Two basically similar ultrasonic systems were employed, each consisting of an *X*-cut quartz transducer having a fundamental thickness resonant frequency of 1 MHz intimately coupled by a thin layer of oil to a planoconcave polystyrene lens. The sound-transmitting medium for all experiments was degassed distilled water. Each system contained a castor oil acoustic absorption chamber, separated from the transmitting medium by an acoustically transparent rubber membrane, in order to assure that all specimens were exposed to traveling-wave fields only. Both systems were provided with mechanical three-coordinate positioning devices enabling the specimen container to be located in the focal volume of the sound beam within  $\pm 0.01$  cm in each orthogonal direction. For System A, the specimen container is moved while the transducer remains fixed in space, and for System B, the specimen container is placed at a fixed position and the transducer is moved. The transducer of System A was  $1\frac{1}{2}$ -in. in diameter and driven by a power amplifier (capable of delivering approximately 8000 V to the quartz plate) controlled by a signal generator. The transducer of System B was  $2\frac{1}{2}$ -in. in diameter and excited by an electronic driver possessing a feedback arrangement to ensure constant predetermined voltage applied to the quartz plate and, consequently, a constant acoustic intensity in the specimen chamber. The focal volume (the volume over which the acoustic intensity is not less than one-half of the peak value) is a function of the sound wavelength<sup>26</sup> and at 1 MHz was determined experimentally to be approximately 0.5 cm in diameter and 1 cm long while at 11 MHz, the corresponding values are 0.05 and 0.3 cm, respectively. With the very high intensities employed with System B, it was necessary to use a pulsing regime in order that the acoustic lens not overheat and alter its focal properties. Thus, an automated arrangement was used whereby the transducer delivered a 0.1-sec pulse every 6 sec. At 11 MHz, complete automation was employed because the combination of small focal volume and short irradiation time required that many pulses be given to ensure that an appreciable fraction of the sample be irradiated. The sample size for this system was 2 ml. Experiments with suspensions of small particles showed that a

0.1-sec pulse resulted in energetic particle motion inside the sample container. Thus, since the acoustic pulse provided good stirring and since there was 5.9 sec between pulses, it appears to be a good assumption that complete mixing occurred after each pulse. With this assumption of complete mixing, a conservative estimate of 2 mm<sup>3</sup> in the focal volume, and a total volume of 2 ml, calculation shows that after 2000 pulses the probability of every particle being in the focal volume at least once is 0.86.

### C. Sample Containers

For System A, where the focal volume at 1 MHz was about 0.8 ml, sample containers were made of Pyrex tubing 32 mm long and 16 mm in i.d. (volume about 6.4 ml). A groove was ground on the outside of each end of the tube to a depth sufficient to hold a  $\frac{1}{16}$ -in.-thick O ring. Both ends of the tube were closed with pieces of 0.0005-in.-thick Saran, which were clamped in place with the O rings. With practice, it is possible to fill this container with degassed sample solution and to close it so that air is not trapped inside. For the vertical System B, the containers were similar but of lesser volume (2.0 ml) and were made of Pyrex tubing of 9-mm i.d. and 32 mm long. The ends were closed in the manner described above. Each type of container was clamped in a small three-fingered laboratory clamp for mounting. The position of the focal volume in the transmitting medium was located by the thermocouple probe method.<sup>26</sup> The acoustic intensity in the focal volume was determined by the radiation pressure technique.<sup>26</sup>

### D. Equipment for Direct Irradiation of Enzyme Activity Assays

An instrument designed especially for the purpose of monitoring the ultrasonically irradiated enzyme-catalyzed reactions was constructed to fit a Beckman model DU spectrophotometer. The cuvette compartment of this spectrophotometer was replaced by an ultrasonic irradiation system consisting of: (a) a 1-MHz *X*-cut quartz transducer, the piezoelectric element which produces ultrasound when excited electrically; (b) a right circular cylindrical reaction cell of stainless steel, volume approximately 20 ml, with quartz windows to transmit the spectrophotometer light beam normal to the direction of ultrasonic wave propagation, and with water from a constant temperature bath circulating through its walls; and (c) an acoustic absorption chamber (filled with castor oil and separated from the reaction cell by a 0.001-in.-thick polyethylene membrane) whose function is to ensure the absence of standing waves by absorbing all incident acoustic energy. The uv light passing through the reaction cell was detected by a 1P28 photomultiplier tube and the electrical output was fed through a Beckman energy recording adapter to a Sargent model SRL recorder which was equipped with logarithmic gears to give a

<sup>26</sup> W. J. Fry and F. Dunn, *Physical Techniques in Biological Research*, W. L. Nastuk, Ed. (Academic Press Inc., New York, 1962), Vol. 4, Chap. 6, pp. 261-394.

plot of absorbance versus time. The slope of the absorbance-versus-time curve gives the rate of the enzyme-catalyzed reaction and was converted, using the appropriate definition for a unit of activity, to the specific activity of the enzyme. More detailed specifications and a description of the construction of the system are given elsewhere.<sup>15</sup>

### E. Degassing Procedure

Below 10 MHz, the acoustic intensity cavitation threshold for water containing dissolved gas is lower than for degassed water.<sup>26</sup> Data for the acoustic-intensity cavitation threshold for most aqueous solutions are not available, but the general principle that the threshold increases with viscosity<sup>27</sup> means that the data for water can be considered minimum values for aqueous solutions more viscous than water. Thus, to avoid cavitation for ultrasonic frequencies below 10 MHz, the sample solution should be degassed and the intensity kept below the acoustic cavitation threshold for degassed water.

Degassing was carried out in a vacuum desiccator using a laboratory vacuum pump. The sample solution, 7–20 ml depending on the sample container size and the number of samples, was placed in a 50-ml Erlenmeyer flask and the flask was covered with a piece of perforated Parafilm to prevent loss of the sample due to the splashing and foaming that occur during degassing. The flask was then placed in the vacuum desiccator and degassed for 10 min. All the visibly detectable degassing was usually completed within 5 min. After 10 min under vacuum, the desiccator was slowly brought to atmospheric pressure, the sample solution was removed and the volume lost due to evaporation of water was replaced by carefully adding degassed distilled water.

## II. EXPERIMENTAL PROCEDURE

### A. Enzymes in Solution: Irradiations and Analyses

The composition of the enzyme solutions studied, the characteristics of the ultrasound to which they were exposed, and the analyses of the irradiated solutions are given in Table I.

The solutions were degassed, the sample containers were filled and positioned in the focal volume of the ultrasonic irradiation systems and for each experiment, a second container was filled and placed in the transmitting medium compartment, but out of the sound beam, to serve as a control. Following irradiation, samples and controls were refrigerated at 5°C until the various analyses could be performed. Appropriate dilutions of aliquots of the irradiated samples and controls were assayed for enzymatic activity and the other properties given in Table I.

The results of the analyses presented in Table I show that there are no significant differences between

<sup>27</sup> T. F. Hueter and R. H. Bolt, *Sonics* (John Wiley & Sons, Inc., New York, 1955), pp. 225–241.

the irradiated samples and their unirradiated controls in any of the experiments and, thus, the enzymes are unaffected in any permanent way by the intense noncavitating ultrasound. A more detailed consideration of each enzyme follows.

Alpha-chymotrypsin is known to be most stable in solution at pH 3–4 and at temperatures in the range 0°–5°C;  $\alpha$ -chymotrypsin solutions of higher pH and solutions stored at higher temperatures undergo gradual autolysis.<sup>28</sup> These facts explain why the specific activities observed for both sample and control in Expts. 3 and 4 (Table I) are less than the corresponding activities at the same pH values but lower temperatures in Expts. 1 and 2. Additional control samples for Expt. 3 (pH 3.0) and Expt. 4 (pH 7.1), which were degassed but not subjected to the 3½-h irradiation time at 37°C showed the same specific activities as the controls for Expt. 2 (pH 3.0) and Expt. 1 (pH 7.1), respectively. The interesting conclusion drawn from these results is that  $\alpha$ -chymotrypsin is not affected by intense noncavitating ultrasound even under conditions that cause gradual denaturation of the enzyme. The  $A_{280\text{ m}\mu}$  and the ratio  $A_{280\text{ m}\mu}/A_{250\text{ m}\mu}$  for 1:100 dilutions of irradiated sample and control are given in Table I in order to compare the absorption spectra. The sample spectra were essentially congruent to those of the controls and this congruence is reflected in the near identity of the  $A_{280\text{ m}\mu}$  and  $A_{280\text{ m}\mu}/A_{250\text{ m}\mu}$  values. In the  $\alpha$ -chymotrypsin experiments, the specific optical rotations of the irradiated samples consistently showed slightly more levorotation than the controls. The accepted value of  $[\alpha]_D$  for  $\alpha$ -chymotrypsin in 0.1M NaCl, pH 3 is  $[\alpha]_D = -66^\circ$ .<sup>29</sup> The discrepancy between this value and the values for the control in Table I is probably mostly due to the difference in the method used for determining concentration, viz., for the value reported in the literature, concentrations were determined by a micro-Kjeldahl method assuming the  $\alpha$ -chymotrypsin to be 16.06% nitrogen; the  $\alpha$ -chymotrypsin concentrations in Table I were determined spectrophotometrically using an absorptivity at 282 m $\mu$  for  $\alpha$ -chymotrypsin of 1.85/cm/mg/ml.<sup>21</sup> However, it is not unusual for  $[\alpha]_D$  values reported by different investigators for proteins under similar conditions to differ slightly.<sup>30</sup> When proteins are denatured, they usually show a large increase in levorotation. If  $\alpha$ -chymotrypsin is denatured with 8M urea in 0.1M NaCl, pH 3,  $[\alpha]_D = -111.6^\circ$ .<sup>29</sup> Therefore, though the small differences between the specific rotations of  $\alpha$ -chymotrypsin samples and controls in Table I were consistently found, because they are small differences they are not interpreted as evidence of denaturation. In the sedimentation velocity analysis of Expt. 1, the irradiated sample and control, which were

<sup>28</sup> M. Kunitz and J. H. Northrup, *J. Gen. Physiol.* 18, 433–458 (1935).

<sup>29</sup> J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg* 30, 450–461 (1953).

<sup>30</sup> P. Urnes and P. Doty, *Advan. Protein Chem.* 16, 401–544 and 486–489 (1961).

TABLE I. Treatment and analyses of ultrasonically irradiated enzyme solutions.

Enzyme	Experiment No.	Cont. (mg/ml)	pH, solvent	Temp. (°C)	Freq. (MHz)	Intensity (W/cm <sup>2</sup> )	Duration	Specific activity <sup>a</sup> (U/mg)		$A_{280\text{ m}\mu}$ (of 1:100 dil.)		$A_{230\text{ m}\mu}/A_{250\text{ m}\mu}$		$[\alpha]_D^{20}$ [at 7°C]		$s_{20,w} \times 10^{18}$ (sec.)	
								Sample	Control	Sample	Control	Sample	Control	Sample	Control		
$\alpha$ -Chymotrypsin	1	10.20	pH 7.1 tris buffer	26.4	1	75	10 min continuous	15 300	15 300	0.185	0.185	2.83	2.83	-54°	-45°	2.63	2.65
	2	10.48	pH 3.0, 0.1M NaCl	23.8	11	1000	1800 0.1-sec pulses	13 500	13 500	0.191	0.191	2.65	2.65	-52°	-48°		
	3	9.62	pH 3.0, 0.1M NaCl	37.0	11	1000	2000 0.1-sec pulses	11 100	11 100	0.177	0.176	2.60	2.67	-53°	-47°		
	4	10.32	pH 7.1 tris buffer	37.0	11	1000	2000 0.1-sec pulses	9 300	9 300	0.191		2.55				2.37	
Trypsin	5	9.05	pH 7.1, tris buffer	20.2	1	75	10-min continuous	4 600	4 600	0.142	0.141	2.60	2.62	-23°	-24°	1.80	1.54
	6	9.24	pH 3.0, 0.1M NaCl	22.7	11	1000	2000 0.1-sec pulses	5 200	5 200	0.145	0.145	2.50	2.59	-48°	-55°		
	7	8.40	pH 3.0, 0.1M NaCl	37.0	11	1000	2000 0.1-sec pulses	3 800	3 800	0.132	0.132	2.60	2.64	-56°	-62°	1.41	1.46
Lactate dehydrogenase	8	8.19	pH 5.8, 17% sat. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	23.0	1	75	10 min continuous	34	34	0.122	0.122	2.60	2.54	-43°	-37°	5.40	5.41
	9	11.20	pH 5.8, 17% sat. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	23.7	11	1000	2000 0.1-sec pulses	29	29	0.167	0.166	1.25	1.29	-38°	-36°		
Aldolase	10	11.00	pH 7.8, 25% sat. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	24.4	11	1000	2000 0.1-sec pulses	11	11	0.100	0.100	3.00	2.94	-20°	-18°	6.26	6.32
	11	9.22	pH 6.8, 0.1M KCl	24.2	11	1000	2000 0.1-sec pulses	1 100	1 100	0.062	0.063	2.10	2.14	-53°	-68°		

<sup>a</sup> Specific activity defined for five enzymes.

U, unit of activity\*

Enzyme

$\alpha$ -Chymotrypsin  $\Delta A_{237\text{ m}\mu}/\text{min} = 0.001, 25^\circ\text{C}, \text{pH } 7.0$

Trypsin  $\Delta A_{237\text{ m}\mu}/\text{min} = 0.001, 25^\circ\text{C}, \text{pH } 7.0$

Lactate dehydrogenase Initial rate of oxidation of 1  $\mu\text{M}$  of NADH<sub>2</sub>/min. 25°C, pH 7.4

Aldolase  $\Delta A_{240\text{ m}\mu}/\text{min} = 1.000, 25^\circ\text{C}, \text{pH } 7.5$

Ribonuclease  $\Delta A_{266\text{ m}\mu}/\text{min} = 0.001, 25^\circ\text{C}, \text{pH } 7.1$

dialyzed versus cold (approximately 5°C), 0.1M NaCl (pH 6.5) before sedimentation, display identical sedimentation behavior; not only are the sedimentation coefficients nearly equal, but the schlieren patterns are congruent. The value of  $s_{20,w}$  is reasonable for the pH and concentration of the solutions.<sup>31</sup> In Expt. 4, the sedimentation coefficient of the irradiated sample was determined in tris buffer, pH 7.1, and, as expected,<sup>31</sup> it is less than the sedimentation coefficients of the sample and control in Expt. 1 at pH 6.5.

The specific activities and uv absorption spectra of the irradiated samples are identical to those of the controls in all experiments with trypsin. The specific optical rotations of samples and controls do not differ significantly and since trypsin is reported to have a  $[\alpha]_D = -40^\circ$  at pH 5.2 and a  $[\alpha]_D = -69^\circ$  at pH 1.3, the values at pH 7.1 and pH 3 given in Table I seem reasonable. The sedimentation coefficients for Expts. 5 and 7 though approximately equal, are not characteristic of native trypsin. The  $s_{20,w}$  for 1% trypsin in tris buffer should be approximately 2.35 S ( $S = 10^{-13}$  sec) and for 1% trypsin in 0.1M NaCl, pH 3,  $s_{20,w}$  should be approximately 2.45 S.<sup>32</sup> The low  $s_{20,w}$  value has been reported before<sup>33</sup> and is attributed to changes in molecular size due to autolysis. The changes in size, however, do not make trypsin susceptible to damage by intense noncavitating ultrasound since the controls were essentially the same as the irradiated samples. In Expt. 7, exposure to a temperature of 37°C lowered the specific activity of trypsin but trypsin did not greatly affect the other properties of the enzyme. This was shown by analysis of a second control for Expt. 7, which was degassed but not subjected to the 3½ h at 37°C. The results of the analyses on this "degassed only" control, for comparison with the results in Table I are: specific activity = 5950  $\Delta A_{237\text{ m}\mu}/\text{min}/\text{mg enzyme}$ ,  $A_{280\text{ m}\mu}$  (of 1:100 dil.) = 0.132,  $A_{280\text{ m}\mu}/A_{250\text{ m}\mu} = 2.68$ ,  $[\alpha]_D^{23^\circ\text{C}} = -58^\circ$ ,  $s_{20,w} = 1.53$  S. The heated control and heated irradiated sample each have 64% of the specific activity of this control and, thus, as with  $\alpha$ -chymotrypsin, intense noncavitating ultrasound did not affect the enzyme even in an environment that gradually denatures it. The sedimentation patterns for experiments revealed that the boundary is asymmetric to the slower sedimenting side of the peak and broadens during sedimentation in each case. This indicates that the enzyme solutions were heterogeneous, as would be expected if autolysis occurred.

The specific activities and uv absorption spectra for irradiated LDH samples and their controls were identical for both experiments in Table I. The irradiated sample and control of Expt. 9 were slightly turbid. This turbidity caused an abnormally high absorption in the

240–260 m $\mu$  region of the uv absorption spectra, which in turn yielded low values for the  $A_{280\text{ m}\mu}/A_{250\text{ m}\mu}$  ratios. The turbid solutions were centrifuged at 24 000 g for 10 min to obtain clear solutions for optical rotation studies. The specific optical rotation results given are close to a reported value of  $-43^\circ$  for LDH in pH 5.6, 19% saturated  $(\text{NH}_4)_2\text{SO}_4$ .<sup>34</sup> The irradiated sample and control of Expt. 8 show the same sedimentation behavior. The solutions were sedimented in 17% saturated  $(\text{NH}_4)_2\text{SO}_4$  and at this high salt concentration, LDH is disassociated into two subunits each of molecular weight approximately 72 000.<sup>35</sup> The  $s_{20,w}$  values found for LDH subunits (at a concentration of 8.19 mg/ml) are in agreement with a reported value of  $s_{20,w} = 5.5$  S (at a concentration of 2.6 mg/ml) for, if the  $s_{20,w}$  of the subunits decreases with increasing concentration (as does undissociated LDH), then an  $s_{20,w}$  at 8.19 mg/ml should be slightly lower than one for a 2.6-mg/ml solution.<sup>35</sup>

The irradiated aldolase sample and its control have identical specific activities and uv absorption spectra. The specific rotations are very nearly equal and are close to a reported value  $[\alpha]_D = -23^\circ$ .<sup>36</sup> The sedimentation behavior of irradiated sample and control (both dialyzed versus 0.1M NaCl, pH 6.5 prior to sedimentation) were also practically identical but the sedimentation coefficients were lower than the value 6.80 S<sup>37</sup> expected for the concentration and pH of the solutions indicating the possibility of a slight expansion of the aldolase molecules. Such an expansion could have occurred since the sample and control were held at 24.4°C for 3½ h during the irradiation. That partial denaturation does occur in the sample and control was shown by analysis of the native solution and a control that was immediately refrigerated after degassing. The results of these analyses are shown in Table II. During degassing, surface denaturation was observed to cause a small amount of flocculent precipitate. The precipitate removed some protein from solution and this is the reason the degassed-only control, heated control, and irradiated sample are slightly less concentrated than the native solution (see  $A_{280\text{ m}\mu}$  results). The surface denaturation also accounts for the specific activity of the degassed only control being lower than that of the native solution. The solutions held at 24.4°C for 3½ h have a lower specific activity than the degassed only control as well as lower sedimentation coefficients but, as with previously discussed enzymes, although aldolase is unstable in the irradiation environment it still was not damaged by intense noncavitating ultrasound.

The results for ribonuclease in Table I, with the exception of the specific optical rotation of the irradiated

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TABLE II. Biochemical analyses of additional aldolase controls.

Aldolase sample	Specific activity ( $\Delta A_{240} \text{ m}\mu/\text{min}/\text{mg enzyme}$ )	$A_{280} \text{ m}\mu$ (1:100 dil.)	$\frac{A_{280} \text{ m}\mu}{A_{250} \text{ m}\mu}$	$S_{20, 40} \times 10^{13}$ (sec)
Degassed only control	12	0.100	3.07	6.48
Native	13	0.104	3.03	6.50

sample, all indicate that the irradiated sample and control solutions were identical. The  $[\alpha]_D = -68^\circ$  observed for the ribonuclease control agrees well with the reported value  $-73.3^\circ$ .<sup>33</sup> Explaining the  $[\alpha]_D$  of the irradiated sample is difficult, because all reported denaturations of ribonuclease, e.g., by oxidation, reduction, high pH, or 8M urea,<sup>30</sup> give substantial increases in levorotation, and this sample exhibits a decrease in levorotation.

Since some of the solutions of Table I were dialyzed prior to sedimentation velocity analysis, it was necessary to determine if amino acids or small peptides were cleaved from the enzyme molecules, either by the ultrasonic treatment or, in the case of trypsin and  $\alpha$ -chymotrypsin, by autolysis. Thus, some of the irradiated samples and controls were analyzed by thin-layer chromatography. The limit of detection for most amino acids by thin-layer chromatography is lower than 0.1  $\mu\text{g}$ .<sup>39</sup> Ten micrograms of each enzyme were applied in small spots to the thin layers and several amino acids in 1- $\mu\text{g}$  quantities were similarly applied for comparison purposes. Trypsin and  $\alpha$ -chymotrypsin solutions gave some faintly ninhydrin positive areas distributed between the origin and approximately two-thirds the distance to the solvent front. The irradiated samples and controls gave identical patterns and color intensities, indicating that only autolysis was responsible for the material detected. None of the other enzymes displayed any evidence of degradation.

### B. Enzyme-Catalyzed Reactions

The following procedure was employed for observing the effects of noncavitating ultrasound on enzyme-catalyzed reactions. The electronic components were tuned up with distilled water in the irradiation cell; the distilled water was then replaced with the degassed substrate for the reaction to be studied. After setting the absorbance at some arbitrarily selected position on the recorder chart, the substrate was irradiated for 1 min, in the absence of enzyme, to determine whether ultrasonic irradiation produced any change in absorbance, i.e., affected the substrate. The temperature was monitored during the irradiation with a thermistor probe inserted in the filling hole of the sample irradiation cell. When assured that irradiation had no effect upon the substrate (other than the effects due to light diffraction or temperature change, which will be discussed later), the enzyme was added to start the reaction. After the reaction had proceeded 1 min, the ultrasound

was turned on for 1 min while the spectrophotometrically monitored rate of reaction,  $\Delta A/l$ , was recorded continuously on the chart paper. After 1 min of ultrasonic irradiation, the ultrasound was turned off and the reaction was allowed to continue as long as the reaction rate remained linear. The temperature was monitored continuously and any changes were noted on the recorder chart. Delivery of the enzyme, a careful stirring of the reaction mixture, and positioning the thermistor probe in the filling hole of the reaction irradiation cell could all be accomplished in 20 sec or less. Degassing the substrate for the 1-MHz irradiations was a precaution taken to ensure the absence of cavitation, although the intensity employed at 1 MHz was below the cavitation threshold for aerated water. If cavitation had occurred in the reaction mixture, it would have been detected because the cavitation bubbles scatter light causing an erratic increase in absorbance.

Several interrelated factors had to be considered in the selection of the procedure outlined above, and the following remarks describe the instrumental limitations from which the procedure evolved. The rate of an enzyme-catalyzed reaction depends on the concentrations of enzyme and substrate and on the temperature and pH of the reaction mixture. Temperature and pH are held constant according to the conditions specified by the definitions for a unit of catalytic activity. The sensitivity of the spectrophotometer limits the range of substrate concentrations that may be used, and within the useful range, preliminary experiments were carried out to determine the best combination of enzyme and substrate concentrations that yields a suitable first-order reaction for study. The criteria for suitability are a reaction whose rate is sufficient to enable detection of a change in rate of 5% or less, and a reaction that remains first order for at least 3 min. Three minutes is considered a minimum time for the reaction to remain first order because it is desirable to be able to observe the reaction for one minute both before and after a 1-min ultrasonic irradiation. Irradiations longer than 1 min usually were undesirable at the intensities employed, since they resulted in temperature increases that could not be brought back to the initial value before the reaction ceased to be first order. Since enzyme-catalyzed reactions are temperature-dependent, approximately doubling in rate for a 10°C temperature increase, it was desirable not only to maintain constant temperature during a given enzyme assay but also to assay at the same temperature when comparing the activities of different samples. If, in addition, the enzyme-catalyzed reaction is ultrasonically irradiated,

<sup>38</sup> F. H. White, Jr., *J. Biol. Chem.* **236**, 1353-1360 (1961).

<sup>39</sup> K. Randerath, *Thin Layer Chromatography* (Academic Press Inc., New York, 1965), pp. 96-97.

## INTENSE NONCAVITATING ULTRASOUND ON ENZYMES

TABLE III. Conditions of the irradiated reactions.

Enzyme	$\mu\text{g}$ Enzyme assayed	Substrate conc.	pH
$\alpha$ -Chymotrypsin	10	$2.89 \times 10^{-4} M$ ATEE	7.0
Trypsin	10	$7.22 \times 10^{-5} M$ BAEE	7.0
Lactate dehydrogenase	0.62	$3.45 \times 10^{-4} M$ Pyruvate	7.4
		$6.90 \times 10^{-5} M$ NADH <sub>2</sub>	
Aldolase	10	$4 \times 10^{-3} M$ Fructose-1, 6-diphosphate	7.3
		$2.3 \times 10^{-2} M$ Hydrazine sulfate	
Ribonuclease	100	0.1 mg/ml cytidine 2':3' phosphate ( $4.68 \times 10^{-4} M$ )	7.1

it is necessary to monitor the temperature throughout the reaction because absorption of ultrasound by the reaction mixture produces a temperature rise. By limiting the irradiation times to 1 min, intensities higher than  $35 \text{ W/cm}^2$  could be employed and the reaction mixture in the thermostated irradiation cell could still be quickly restored to its initial temperature after irradiation.

Two phenomena that complicated the interpretation of the results were a substrate absorbance change with increased temperature and the Debye-Sears effect.

Temperature increases, due to acoustic absorption, changed the light absorbance ( $\Delta A/\Delta T$ ) of solutions of ATEE, BAEE, cytidine 2':3' cyclic phosphate and solutions of their reaction products. For the maximum temperature increases produced during irradiation, the observed  $\Delta A$  was approximately 5% of the total change observed in the assay. However, since the original temperature was quickly re-established after the acoustic exposure and since irradiation of substrate alone had previously provided knowledge of the time course of  $\Delta A/\Delta T$ , the effects of  $\Delta A/\Delta T$  were readily separated from other ultrasonic effects on the reaction rate. It was particularly easy to detect the effect of  $\Delta A/\Delta T$  in the case of the  $\alpha$ -chymotrypsin assay since the assay produces a decrease of  $A_{237 \text{ m}\mu}$  while  $\Delta A/\Delta T$  produces an increase.

The diffraction of light passing through a transparent medium that is also transmitting a beam of parallel ultrasonic waves is known as the Debye-Sears effect.<sup>27</sup> Diffraction occurs as a result of the slight differences in refractive index between the alternate regions of compression and rarefaction in the medium and, hence, the ultrasonic waves may be considered a three dimensional diffraction grating traveling with the speed of sound. However, since the speed of light is much greater than the speed of sound, the diffraction grating is effectively stationary. In the resulting diffraction pattern, the light intensity of the zeroth-order line is inversely proportional to the acoustic intensity, i.e., the greater the acoustic intensity, the greater is the proportion of light diverted from the zeroth-order to higher orders.<sup>10</sup> Debye-Sears diffraction could affect the form of the experimental results, since the acoustic intensity is sufficient to diffract light into higher orders that do not reach the detector and the result on the recorder

<sup>10</sup>J. Blitz, *Fundamentals of Ultrasound* [Butterworth and Co. (Publishers) Ltd., London, 1963], pp. 137-141.

chart is an apparent increase in absorption. However, these diffraction effects presented no difficulty in the interpretation of data in this study, since they merely produce an abrupt displacement of the absorbance to a higher level marking the commencement of ultrasonic irradiation. The reaction rate,  $\Delta A/t$ , continues to be recorded, but displaced by a fixed amount. When the ultrasound was turned off, the absorbance abruptly decreases by this same amount while the reaction continues.

Table III gives the amount of enzyme, the substrate concentration, and the pH in the ultrasonically irradiated reaction mixture.

The results of the ultrasonic irradiations of the enzyme-catalyzed reactions are listed, according to the frequency and intensity employed, in Table IV. All the irradiations were continuous for 1 min and the sample irradiation cell was maintained, except for the brief temperature rise indicated, at  $25.0 \pm 0.1^\circ\text{C}$ . The 1-MHz ultrasound and the lowest intensity ( $0.5 \text{ W/cm}^2$ ) at 9 MHz (for the first four enzymes of Table V) were generated by a crystal with a 1-MHz fundamental resonant frequency. The higher intensities at 9 and 27 MHz were generated by a crystal with a 9-MHz fundamental resonant frequency.

It is seen that none of these irradiations (Table IV) had any effect on the enzyme-catalyzed reactions.

### III. CONCLUSIONS

The results of this study lead to the following conclusions. Irradiation of approximately 1% solutions of  $\alpha$ -chymotrypsin, trypsin, lactate dehydrogenase, aldolase, and ribonuclease with noncavitating ultrasound at dose levels sufficient to cause extensive structural and functional damage in tissues, has no effect on either the structure or function of the enzymes. That the catalytic function of the five enzymes was unaffected by intense noncavitating ultrasound was demonstrated in two ways: The approximately 1% solutions showed full catalytic ability when assayed after irradiation and irradiations failed to inhibit the enzymes while they were in the process of catalyzing a reaction. Structural integrity of the irradiated enzyme samples was demonstrated by the lack of any significant differences between the uv absorption spectra, specific rotations, sedimentation coefficients, and thin-layer chromatographic analyses of the irradiated samples and unirradiated samples.

TABLE IV. Effects of intense noncavitating ultrasound on enzyme-catalyzed reactions (1-min continuous irradiations at 25°C).

Enzyme of the irradiated reaction	Frequency (MHz)	Intensity (W/cm <sup>2</sup> )	Max. temp. rise during irradiation (°C)	% Native activity
α-Chymotrypsin	1	5	0.5	100
	9	0.5, 3, 23	<0.1, 0.5, 2.5	100 100 100
	27	1	1.0	100
Trypsin	1	5	0.5	100
	9	0.5, 4, 23	<0.1, 0.7, 3.3	100 100 100
	27	1	1.0	100
Lactate dehydrogenase	1	5	0.5	100
	9	0.5, 14, 39	<0.1, 0.7, 1.5	100 100 100
	27	1	1.2	100
Aldolase	1	5	0.5	100
	9	0.5, 12, 37	<0.1, 0.8, 1.5	100 100 100
	27	1	1.2	100
Ribonuclease	9	14, 39	0.8, 1.7	100 100
	27	1	1.0	100

radiated controls. Since the denaturation of the enzymes studied in this paper by cavitating ultrasound has been shown in other reports,<sup>41</sup> it is concluded that cavitation is a necessary condition for damage to be produced by ultrasound in the five enzyme solutions *in vitro* of this study.

These conclusions raise several points worthy of further discussion. *First*, the inability of intense noncavitating ultrasound to damage the enzymes, particularly trypsin solutions, contradicts the findings of other investigators.<sup>9</sup> A recent study<sup>42</sup> carried out in conjunction with the work reported here has shown that the reported inactivation of trypsin<sup>9</sup> and α-amylase<sup>10</sup> were not due directly to an interaction between the ultrasonic waves and the protein molecules in solutions, but rather to an unspecified reaction between the solution and rubber materials employed in the specimen container.

Another interesting aspect of the results obtained with intense noncavitating ultrasound is the fact that, despite the expected absorption of acoustic energy being many times greater than the energy required to denature the enzymes, the mechanisms of absorption and dissipation of this energy apparently do not involve permanent changes in the enzyme molecules nor detectable transient changes that in any way affect the normal functioning of the enzyme *in vitro*. The results suggest that the mechanism of acoustic energy absorption does not involve great changes in the secondary and tertiary structure of the enzyme molecules, and this information may be useful to investigators studying the mechanisms of acoustic absorption by polymers in solution.

The molecular biological approach in studying the interactions of intense noncavitating ultrasound with biological structures has revealed that DNA<sup>41</sup> can be degraded *in vitro*, but enzymes cannot. Degradation of DNA *in vivo* cannot account for the effects observed in

irradiated tissues since it is unlikely that the loss of cellular control processes involving DNA would result in the rapid functional changes observed. The inactivation of enzymes would provide a more reasonable explanation for the rapidly appearing effects in tissues since they are more directly concerned in the chemical reactions essential to the cell's survival. The fact that intense noncavitating ultrasound has no effect on enzymes *in vitro* does not necessarily imply that there are no interactions in the cellular environment. Some enzymes, such as the enzymes of the Krebs cycle (which are located in mitochondria), apparently are structurally organized in groups. In the case of the Krebs cycle, the enzymes are closely associated with, and possibly bound to, the cristae of the mitochondria.<sup>43</sup> Thus, it is possible that in this structured environment, an enzyme may be more susceptible to denaturation by intense noncavitating ultrasound either directly or by some indirect mechanism involving the adjacent structures. Use of histochemical techniques<sup>44</sup> may prove useful for future studies of the effects of intense noncavitating ultrasound on enzymes *in vivo*.

It is felt that the most promising future research into the nature of the interactions between intense noncavitating ultrasound and tissue will deal with those levels of biological structure that lie between the molecular level and structures observable with the light microscope. A study of the effects of intense noncavitating ultrasound on the properties of membranes would be useful not only because membranes are a ubiquitous structural feature of cells, being found around the nucleus and as a part of such cellular organelles as mitochondria, endoplasmic reticulum, etc., but also because of the importance of excitable membranes in nervous tissue.

#### ACKNOWLEDGMENTS

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