coastal waters, the fish are, on the average, larger, restricted to a lesser depth, and much more numerous.

This discussion shows that the mode-stripping model in its present form is generally insufficient to explain the experimental results. Attempts to deduce mode-attenuation coefficients and parameters, such as bottom-loss factor, are likely to be unsuccessful. The theory is not so much wrong as incomplete; and there may well be special circumstances when the unmodified theory *does* work. These might include very short ranges, especially at the lower frequencies and over rocky bottoms, and abnormal propagation such as that which occurs during storms or under ridged ice. Although typical attenuation rates have been quoted, it must be borne in mind that one of the chief features of shallow-water transmission is its variability.

A subsidiary conclusion of this letter is the importance of knowing the effective number of normal modes, in order to understand shallow water propagation. The number of modes may be discovered from the time dispersion among the mode arrivals (e.g., sonagram analysis); from the interference patterns between the modes as a function of range, depth, or frequency; or from the gross wide-band depth dependence of intensity. The first two approaches give information on both the magnitude and the phase (or coherence) of the modes; the last approach is not sensitive to phase.

The main conclusion is the failure of the simple mode-stripping theory, probably because the transmission is overwhelmed by a large bulk attenuation, which in turn is very likely due to fish. Even if the present connection with fish is eventually disproved, the general importance of fish attenuation in shallow water must not be discounted.

[1] D. E. Weston, "Propagation of Sound in Shallow Water," J. Brit. IRE 26, 329–337 (1963).
[2] D. E. Weston, "Sound Propagation in the Presence of Bladder Fish," in *Underwater Acoustics, Volume 2, Proceedings of the Copenhagen Institute*, V. M. Albers, Ed. (Plenum Press, New York, 1967), Chap. 5.
[3] A. C. Kibblewhite and R. N. Denham, "Experiment on Sound Propagation in Shallow Water under Isovelocity Conditions," J. Acoust. Soc. Am. 40, 1337–1344 (1966).
[4] W. H. Thorp, "Deep-Ocean Sound Attenuation in the Sub- and Low-Kilocycle-per-Second Region," J. Acoust. Soc. Am. 38, 648–654 (1965).
[5] D. E. Weston, "Fish as a Possible Cause of Low-Frequency Acoustic Attenuation in Deep Water," J. Acoust. Soc. Am. 40, 1558 (L) (1966).

16.2 Received 3 April 1967

Effects of Ultransonic Cavitation on Trypsin, α-Chymotrypsin, and Lactate Dehydrogenase Solutions*

R. M. MACLEOD† AND F. DUNN

Biophysical Research Laboratory, University of Illinois, Urbana 61801

The denaturation of trypsin solutions by ultrasonic cavitation, reported by other investigators, has been confirmed. Similar studies on solutions of α-chymotrypsin and lactate dehydrogenase demonstrate that these enzymes are also denatured by ultrasonic cavitation.

THE EFFECTS OF ULTRASONIC CAVITATION ON MANY ENZYME SOLUTIONS have been reviewed recently.[1] It is apparent that many types of instrumentation are employed that produce appreciably different ultrasonic field configurations within the liquid specimen. Further, greatly differing handling and treatment procedures are used depending upon the properties of the enzyme studied. Nevertheless, the prevailing attitude implicit in most studies seems to be that the acoustical parameters are relatively unimportant, provided cavitation is produced. Though most investigators appear to have been content to demonstrate enzyme inactivation in the presence of cavitation, recently,[2–4] the assessment of the molecular damage responsible for the observed enzyme inactivation has become a topic of concern. Since cavitating ultrasound is growing in importance as an intial step in procedures for extracting biological macromolecules and larger structures from cells, it is important for all investigators using cavitation to understand and appreciate the possibility of producing artifacts. Although the work reported in the present paper did not allow specification of the ultrasonic field parameters, the results of the analyses are considered to be of value to those requiring ultrasonic treatment of solutions that contain proteins. This report presents the results of physical and chemical analyses of solutions of trypsin, α-chymotrypsin, and lactate dehydrogenase (LDH) after various exposures to cavitating ultrasound that were performed in conjunction with a wider study on the effects of noncavitating ultrasound on protein solutions.[5]

Trypsin (2× crystallized), α-chymotrypsin (3× crystallized), and LDH (2× crystallized from rabbit muscle) were obtained from the Worthington Biochemical Corporation. Trypsin,[6] α-chymotrypsin,[6] and LDH[7] 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. Cavitating ultrasound was produced by a Branson Sonifier (20 kc/sec) model LS-75, equipped with a "microtip." All experiments were done at power setting No. 2, the highest power setting being No. 8. At power settings greater than 2, cavitation was so vigorous that much of the sample was splashed out of the container, even though it was partially covered. Samples of 4 ml each were cavitated in stainless-steel Morton culture tube closures (17.5 mm i.d.×37.5 mm long) covered with Parafilm with a hole through which the "microtip" was inserted into the sample solution. The containers were kept immersed in an ice-water bath (0°C). The long irradiations were divided into a series of short exposures (none longer than 3 min) so that the sample temperature was always maintained below 20°C. Temperatures were measured with a thermistor within 5 sec after an irradiation. Sedimentation velocity experiments were performed with a Spinco model E analytical ultracentrifuge, equipped with schlieren optics and a rotor temperature indicator and control (RTIC) unit. A valve-type synthetic boundary cell was used in an An-D rotor at a speed of 56 100 rpm. Specific optical rotations, $[\alpha]_D^{T°C}$, were determined with a Rudolph model 70 precision polarimeter using a 10.00-cm microtube (0.7 ml). A Cary model 14 automatic scanning and recording spectrophotometer was used to determine ultraviolet absorption spectra. Thin-layer chromatography experiments were performed using the Eastman Chromagram developing apparatus and Eastman Chromagram sheets, type K301R. Samples were spotted on the chromatograms with lambda pipets. The solvent system used was *n*-butanol; acetic acid; water (3:1:1). After development and drying, spots were located by spraying with ninhydrin, 0.3% in absolute ethanol, and heating at 90°C for 15 min.

All experiments were carried out with the free boundary of the solution in contact with air, i.e., in the presence of oxygen. Control samples were treated exactly as the ultrasonically irradiated samples, except for the induction of cavitation. Table I shows the effects of cavitation on enzyme activity. Enzyme-activity loss, as a function of cavitation time, is shown in Fig. 1 for the 0.95-mg/ml α-chymotrypsin solution and the 0.62-mg/ml LDH solution. The 5 min-cavitated α-chymotrypsin sample and a control sample were also assayed at substrate concentrations one-half and twice the amount used for the assays of Table I, and the results of the assays are plotted in the form suggested by Lineweaver and Burk[8] in Fig. 2. For the substrate used (*N*-acetyl-L-tyrosine ethyl ester), the Michaelis constant K_m has been shown to approximate a true dissociation constant for the enzyme substrate complex,[9] and thus, K_m determination provides a measure of enzyme-substrate affinity that is useful in assessing damage to the enzyme molecules. Table II gives the specific optical rotation and sedimentation coefficient for the control, and for the 15 min-cavitated sample of the 6.20-mg/ml LDH solution.

TABLE I. Effects of 20-kHz ultrasonic cavitation on enzyme activity.

Enzyme	Conc. (mg ml)	Solvent	pH	Irradiation time (min)	% Control activity
Trypsin	0.92	Tris Buffer	7.1	30	69
α-chymotrypsin	10.20	Tris Buffer	7.1	1	100
	10.20	Tris Buffer	7.1	5	100
	10.20	Tris Buffer	7.1	10	100
	0.95	Tris Buffer	7.1	1	94
	0.95	Tris Buffer	7.1	5	71
	0.95	Tris Buffer	7.1	15	69
Lactate dehydrogenase	6.20	17% Sat. $(NH_4)_2SO_4$	5.8	15	37
	0.62	1.7% Sat. $(NH_4)_2SO_4$	6.5	1	89
	0.62	1.7% Sat. $(NH_4)_2SO_4$	6.5	5	61
	0.62	1.7% Sat. $(NH_4)_2SO_4$	6.5	15	25
	0.62	1.7% Sat. $(NH_4)_2SO_4$	6.5	21	11
	0.62	1.7% Sat. $(NH_4)_2SO_4$	6.5	30	4

Precipitation of denatured protein was observed in all the cavitated samples of Table I that showed activity loss. The uv-absorption spectra of the samples were determined after removing the precipitate by centrifugation or filtration. The postcavitation turbidity in the 0.92-mg/ml trypsin and 0.95-mg/ml α-chymotrypsin samples was slight, and difficult to detect by eye. Centrifugation of the samples revealed small amounts of a cloudy gellike precipitate, and the more extensively degraded samples yielded a greater amount of the precipitate. The flocculent precipitate in the LDH samples was removed by filtration. The shape of the uv-absorption spectra of the inactivated samples was the same as that of the untreated controls, though the absorption amplitude was decreased as expected, since the precipitated protein had been removed previously. However, the absorbance at 280 mμ does not decrease to the same extent that the activity does, indicating that the precipitate-free cavitated samples contain inactive enzyme, i.e., not all the inactive enzyme precipitates.

The cavitation of 0.92-mg/ml trypsin for 30 min resulted in an activity loss of 31%. El'piner *et al.*[2] observed an activity loss of 85% after cavitating 0.20-mg/ml trypsin for 30 min. The differences in equipment, procedure, concentration, pH, and method of activity assay, make more than a qualitative comparison impossible. However, the fact that α-chymotrypsin (an enzyme very similar to trypsin) appears to be more susceptible to cavitation denaturation as the concentration decreases (see Table I), may mean that this same factor (lower concentration) is principally responsible for the greater inactivation of trypsin observed by El'piner and co-workers. El'piner *et al.*[2] did not report precipitation of denatured trypsin. However, since precipitation was only barely visible at a concentration of 0.92-mg/ml (Table II), at a concentration only about one-fifth this amount, turbidity may have existed and not been detected by the Russian investigators. If the trypsin solutions of El'piner *et al.*[2] were slightly turbid, the over-all uv-absorption increase that they reported, whose magnitude depended on cavitation time, could be due to light scattering from aggregates of denatured trypsin.

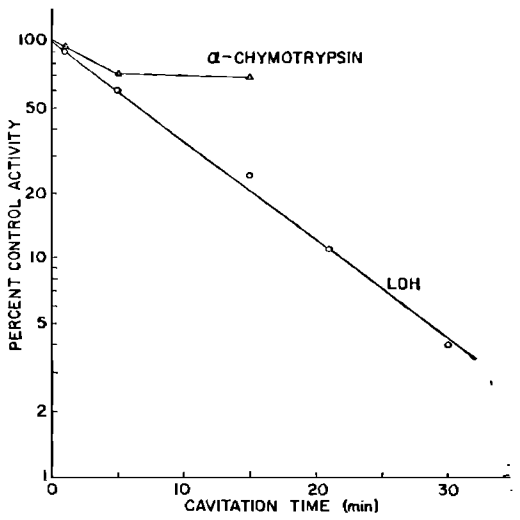

FIG. 1. Enzyme activity versus cavitation time for 0.95-mg/ml α-chymotrypsin solution and for 0.62-mg/ml lactate-dehydrogenase solution.

Figure 1 shows that the activity loss for the 0.62-mg/ml LDH solution exposed to cavitation is given by the equation $A = A_0 e^{-kt}$, where A is the enzyme activity after cavitation for time t, A_0 is the initial activity ($t=0$), and $k=0.105$ (determined from the data of Fig. 1). Cavitation is known to produce free radicals in aqueous solutions,[10] and thus, the fact that the inactivation kinetics follow the same equation as enzyme solutions irradiated with x rays suggests that the cause of enzyme inactivation, in the case of cavitation, may be the same as that for ionizing radiation, viz., the chemical effects of free radicals.

LDH was also cavitated for 15 min at a concentration of 6.20 mg/ml, and activity loss, precipitation of denatured enzyme and decrease in uv absorption, resulted (Table I). After removing the precipitate, the specific rotation and the sedimentation coefficient were determined and compared to values obtained for the control sample (Table II). The increase in levorotation indicates the presence of some denatured enzyme in the filtrate of the irradiated sample, although the $[\alpha]_D$ was calculated using a spectrophotometrically determined concentration for the filtrate and, if the filtrate contains molecules with altered absorptivities, the concentration (and, hence, the calculated $[\alpha]_0$) may be in error. The sedimentation coefficient of the cavitated LDH is considerably reduced, indicating either fragmentation to smaller molecules or an unfolding that increases the frictional coefficient of the molecules. Since the 15-min-cavitated sample possessed 37% native activity, and no peak appeared in its schlieren pattern with a native S value, it seems likely that the peak in the cavitated sample's schlieren pattern represents intact but unfolded molecules with 37% native LDH activity. The alternative conclusion is that the peak represents a nearly 100% conversion of 5.78-S molecules into 3.94-S fragments, and that these uniform fragments possess 37% of the activity of the native molecules.

TABLE II. Properties of cavitated 6.20-mg/ml LDH.

	$[\alpha]_D$	$S_{20,w}$
Control	−38°	5.78
15-min sample	−50°	3.94

α-chymotrypsin at a concentration of 10.20 mg/ml is completely resistant to the cavitation employed in these experiments (Table I), while the inactivation of the 0.95-mg/ml α-chymotrypsin solution is exponential for 5 min and then becomes more resistant to inactivation (Fig. 1). This apparent concentration dependence deserves further investigation, however, in the absence of data at other enzyme concentrations no explanation can be offered. The 0.95-mg/ml α-chymotrypsin results might be interpreted to mean that after 5 min the enzyme was degraded as far as was possible, under these irradiation conditions, and that the solution contained enzyme molecules each with 70% of its original activity. This possibility seems to be ruled out by the results shown in Fig. 2, for if the enzyme molecules are damaged but partially active, it is expected that their affinity for substrate molecules will be reduced and, hence, that their K_m will be greater than the K_m of the native enzyme. Figure 2 shows that the 5-min-

cavitated α-chymotrypsin has the native K_m but a reduced maximum velocity, indicating that fully active enzyme is present only in reduced amount.

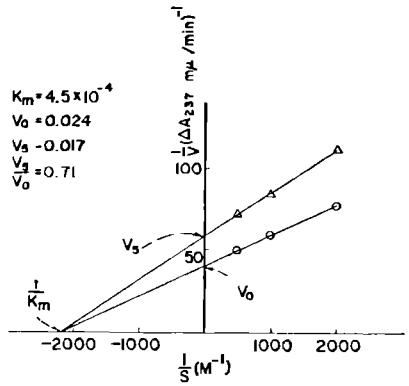

FIG. 2. Lineweaver Burk plot for control and for 5-min-cavitated 0.95-mg/ml α-chymotrypsin samples.

Thin-layer chromatography was done on the 15 min-cavitated 6.20-mg/ml LDH solution, the 30 min cavitated 0.95-mg/ml α-chmotrypsin solution, the 30 min-cavitated 0.92-mg/ml trypsin solution, and appropriate controls. Five micrograms of each sample were analyzed, and no amino acids or small peptides were detected in any of the samples.

The results of this study confirm the previous finding[2] that cavitating ultrasound denatures trypsin in solution and show that LDH solutions—and, under proper conditions, α-chymotrypsin solutions—are also inactivated by cavitating ultrasound. Further, the studies of some of the kinetic and physicochemical properties of α-chymotrypsin and LDH provide additional details concerning the inactivation of enzymes in solution by cavitating ultrasound.

Acknowledgments: This work was supported by the National Science Foundation and the Office of Naval Research, Acoustics Programs.

Extracted from the thesis submitted by R. M. Macleod in partial fulfillment of the requirements for the PhD degree in biophysics, Univ. of Illinois, Urbana, Ill., 1966.

† Present address: Dept. of Biochem., School of Basic Med. Sci., Univ. of Tennessee, Memphis, Tenn.

[1] I. E. El'piner, *Ultrasound: Physical, Chemical and Biological Effects* (Consultants Bureau, Inc., New York, 1964), pp. 149–231.
[2] I. E. El'piner, G. A. Deborin, and O. M. Zorina, Biokhim. 24, 817–822 (1959).
[3] I. E. El'piner and O. M. Zorina, Biophysics (USSR) 5, 649–655 (1960).
[4] L. M. Bronskaya and I. E. El'piner, Biophysics (USSR) 8, 344–348 (1963).
[5] R. M. Macleod, PhD thesis in biophysics, Univ. of Illinois, Urbana, Ill., 1966.
[6] G. W. Schwert and Y. Takenaka, Biochim. Biophys. Acta. 16, 570–582 (1955).
[7] A. Kornberg, in *Methods in Enzymology*, S. P. Colowick, and N. O. Kaplan, Eds. (Academic Press Inc., New York, 1955), Vol. 1, p. 441–443.
[8] H. Lineweaver and D. Burk, J. Am. Chem. Soc. 56, 658–666 (1956).
[9] L. W. Cunningham and C. S. Brown, J. Biol. Chem, 221 287–299 (1956).
[10] A. Henglein, Makromol. Chem. 15, 188–210 (1955).

Errata: Axisymmetric Plane-Strain Vibrations of a Thick-Layered Orthotropic Cylindrical Shell [J. Acoust. Soc. Am. 40, 1509–1516 (1966)]

NAZEER AHMED

Department of Theoretical and Applied Mechanics, Cornell University, Ithaca, New York 14850

The following equations contain errors and are given here in their correct form:

In Eq. 10, b should be replaced by κ.

Equation 15 should be

$$
\begin{aligned}
&-P_{m-1}\Big[J_\beta(\lambda_m r_m)\Big\{e_m\Gamma_\beta'(\lambda_m r_m)-\frac{\kappa_m\Gamma_\beta(\lambda_m r_m)}{r_m}\Big\}\\
&-\Gamma_\beta(\lambda_m r_m)\Big\{e_m J_\beta'(\lambda_m r_m)-\frac{\kappa_m J_\beta(\lambda_m r_m)}{r_m}\Big\}\Big]/D_m\\
&+P_m\Big[J_\beta(\lambda_m r_m)\Big\{e_m\Gamma_\beta'(\lambda_m r_{m-1})-\frac{\kappa_m\Gamma_\beta(\lambda_m r_{m-1})}{r_{m-1}}\Big\}\\
&-\Gamma_\beta(\lambda_m r_m)\Big\{e_m J_\beta'(\lambda_m r_{m-1})-\frac{\kappa_m J_\beta(\lambda_m r_{m-1})}{r_{m-1}}\Big\}\Big]/D_m\\
&+P_m\Big[J_\beta(\lambda_{m+1}r_m)\Big\{e_{m+1}\Gamma_\beta'(\lambda_{m+1}r_{m+1})-\frac{\kappa_{m+1}\Gamma_\beta(\lambda_{m+1}r_{m+1})}{r_{m+1}}\Big\}\\
&-\Gamma_\beta(\lambda_{m+1}r_m)\Big\{e_{m+1}J_\beta'(\lambda_{m+1}r_{m+1})-\frac{\kappa_{m+1}J_\beta(\lambda_{m+1}r_{m+1})}{r_{m+1}}\Big\}\Big]/D_{m+1}\\
&-P_{m+1}\Big[J_\beta(\lambda_{m+1}r_m)\Big\{e_{m+1}\Gamma_\beta'(\lambda_{m+1}r_m)-\frac{\kappa_{m+1}\Gamma_\beta(\lambda_{m+1}r_m)}{r_m}\Big\}\\
&-\Gamma_\beta(\lambda_{m+1}r_m)\Big\{e_{m+1}J_\beta'(\lambda_{m+1}r_m)-\frac{\kappa_{m+1}J_\beta(\lambda_{m+1}r_m)}{r_m}\Big\}\Big]/D_{m+1}=0.
\end{aligned}
$$

Equations 31 and 32 should be

$$R = R(r_{m+1}) - R(r_m),$$

where

$$
\begin{aligned}
R(r) = \sum_m \Big[&\tfrac{1}{2}\rho_m q_m{}^2 r^2\{.1_{lm}{}^2[J_\beta'^2(\lambda_{lm}r)+J_\beta{}^2(\lambda_{lm}r)(1-\beta_m{}^2/r^2\lambda_{lm}{}^2)]\\
&+B_{lm}{}^2[\Gamma_\beta'^2(\lambda_{lm}r)+\Gamma_\beta{}^2(\lambda_{lm}r)(1-\beta_m{}^2/r^2\lambda_{lm}{}^2)]\\
&+.1_{lm}B_{lm}J_\beta(\lambda_{lm}r)\Gamma_\beta(\lambda_{lm}r)-\tfrac{1}{2}.1_{lm}B_{lm}J_{\beta-1}(\lambda_{lm}r)\Gamma_{\beta+1}(\lambda_{lm}r)\\
&-\tfrac{1}{2}.1_{lm}B_{lm}J_{\beta+1}(\lambda_{lm}r)\Gamma_{\beta-1}(\lambda_{lm}r)\}\Big]/\\
&\sum_m\{.1_{lm}r_s J_\beta(\lambda_{lm}r_s)+B_{lm}r_s\Gamma_\beta(\lambda_{lm}r_s)\}.
\end{aligned}
$$

In Eq. 34, the order of the Bessel functions J and Y should be β, not 1.

In Eq. 36, the quantity e_2/e_1 should be replaced by (ψ_1/ψ_2), where ψ_1 and ψ_2 are functions of the elastic constants, densities, and inner and outer radii of the inner and outer layer, respectively.

Erratum: Atmospheric Effects on Sonic Booms [J. Acoust. Soc. Am. 41, 522–523(L) (1967)]

WALTON L. HOWES

NASA-Lewis Research Center, Cleveland, Ohio 44135

Insert 2^{-1} on the right side of Eq. 10.