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., sonogram 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.


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

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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. 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 implied 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, 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 initial 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.

Trypsin (2X crystallized), α-chymotrypsin (3X crystallized), and LDH (2X crystallized from rabbit muscle) were obtained from the Worthington Biochemical Corporation. Trypsin, α-chymotrypsin, and LDH 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 water 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 thermometer 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 86 100 rpm. Specific optical rotations, [α], 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 lambsk PIPEts. The solvent system used was n-butanol; acetic acid; water (5:1:1). After development and drying, spots were located by spraying with ninhydrin, 0.3 ml 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 0.35-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 Lineaweaver and Burk in Fig. 2. For the substrate used (3-acetyl-L-tyrosine ethyl ester), the Michaelis constant Km has been shown to approximate a true dissociation constant for the enzyme-substrate complex. A Km determination provides a measure of enzyme-substrate affinity which 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.

The Journal of the Acoustical Society of America 527
Precipitation of denatured protein was observed in all the cavi-
tated 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 postcavitatiou
absorption spectra of the samples were determined after removing
the LDH samples was removed by filtration. The shape of the
greater amount of the precipitate. The flocculent precipitate in
tion of the samples revealed small amounts of a cloudy gellike
sin samples was slight, and difficult to detect by eye. Centrifuga-
ated samples of Table I that showed activity loss. The uv~
uv-absorptlon spectra of the inactivated samples was the same as
removed previously. However, the absorbance at 280 m does not
possible. However, the fact that a-chymotrypsin (an enzyme very'
activity assay, make more than a qualitative comparison im-
exist on cavitation time, could be due to light scattering from
aggregates of denatured trypsin. 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, 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,
vis., the chemical effects of free radicals.
LDH was also cavitatived 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 spectrophotomet-
rically determined concentration for the filtrate and, if the
filtrate contains molecules with altered absorptivities, the concen-
tration (and, hence, the calculated \([\alpha]_D \)) may be in error. The sedi-
mentation coefficient of the cavitated LDH is considerably re-
duced, 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 repres-
ents a nearly 100% conversion of 3.78-S molecules into 3.94-S frag-
ments, and that these uniform fragments possess 37% of the
activity of the native molecules.

a-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 a-chymotryp-
sin solution is exponential for 5 min and then becomes more re-
sistant to inactivation (Fig. 1). This apparent concentration de-
pendence deserves further investigation, however, in the absence
of data at other enzyme concentrations no explanation can be
offered. The 0.95-mg/ml a-chymotrypsin results might be inter-
preted 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 par-
tially active, it is expected that their affinity for substrate mole-
cules 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-

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Conc. (mg/ml)</th>
<th>Solvent</th>
<th>pH</th>
<th>Irradiation time (min)</th>
<th>% Control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>0.92</td>
<td>Tris Buffer</td>
<td>7.1</td>
<td>30</td>
<td>69</td>
</tr>
<tr>
<td>a-Chymotrypsin</td>
<td>10.20</td>
<td>Tris Buffer</td>
<td>7.1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10.20</td>
<td>Tris Buffer</td>
<td>7.1</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.95</td>
<td>Tris Buffer</td>
<td>7.1</td>
<td>1</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>0.95</td>
<td>Tris Buffer</td>
<td>7.1</td>
<td>5</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>0.95</td>
<td>Tris Buffer</td>
<td>7.1</td>
<td>15</td>
<td>69</td>
</tr>
<tr>
<td>Lactate Dehydrogenase</td>
<td>6.20</td>
<td>17% Sat. (NH4)SO4</td>
<td>5.8</td>
<td>15</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>(NH4)SO4</td>
<td>6.5</td>
<td>1</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>(NH4)SO4</td>
<td>6.5</td>
<td>5</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>(NH4)SO4</td>
<td>6.5</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>(NH4)SO4</td>
<td>6.5</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>(NH4)SO4</td>
<td>6.5</td>
<td>30</td>
<td>4</td>
</tr>
</tbody>
</table>

### Table II. Properties of cavitated 6.20 mg/ml LDH.

<table>
<thead>
<tr>
<th>Control</th>
<th>[(\alpha)]_D</th>
<th>S_{m,0}</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-min sample</td>
<td>(-38^o)</td>
<td>5.78</td>
</tr>
<tr>
<td></td>
<td>(-39^o)</td>
<td>3.94</td>
</tr>
</tbody>
</table>

**Fig. 1.** Enzyme activity versus cavitation time for 0.95 mg/ml a-
chymotrypsin solution and for 0.05 mg/ml lactate-dehydrogenase solution.
cavitated α-chymotrypsin has the native $K_m$ but a reduced maximum velocity, indicating that fully active enzyme is present only in reduced amount.

$$K_m = 4.5 \times 10^{-5}$$
$$V_o = 0.024$$
$$V = 0.007$$
$$V_o = 3.71$$

Thin-layer chromatography was done on the 15 min-cavitat ed 0.20-mg/ml LDH solution, the 30 min cavitat ed 0.95-mg/ml α-chymotrypsin solution, the 30 min-cavitat ed 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 that cavitating ultrasound denatures trypsin in solution and show that LDH solutions and, under proper conditions, alpha-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.

Errata: Axisymmetric Plane-Strain Vibrations of a Thick-Layered Orthotropic Cylindrical Shell

Erratum: Atmospheric Effects on Sonic Booms

Figure 1. Lineweaver-Burk plot for control and for 5-min-cavitat ed 0.95-

mg/ml α-chymotrypsin samples.