

THE DENATURATION OF PROTEINS BY SOUND WAVES OF AUDIBLE FREQUENCIES

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When egg albumin in aqueous solution at the isoelectric point is subjected to the action of intense sound waves of audible frequency, a coagulum is produced (1). A previous investigation (2) indicated that such sound-denatured¹ albumin is immunologically similar to denatured albumin prepared in other ways; *e.g.*, by heat in acid, alkaline, or neutral solution, by alcohol, or by acid or alkali.

Wu and Liu (3), making use of ultrasonic waves of much higher frequency than any employed by us, were able to coagulate albumin. However, when CO₂ or H₂S was substituted for air in the solutions or when no gas at all was present, they observed no change with ultrasonic treatment. This contrasts with their observation that the reaction proceeds normally in the presence of pure O₂ or H₂.

Although the results of our previous studies indicate that the end-product of sonic denaturation is probably the same as that produced by heating and therefore may be the result of a thermal type of activation, they leave unexplained the mechanism through which mechanical vibrations may energize the reaction. The peculiar relationship of the reaction toward certain gases reported by Wu and Liu has not clarified the mechanism for either range of frequency.

We have therefore undertaken a further study of the sonic denaturation of certain proteins, first, to compare the end-products of the reactions with those produced by other means with

¹ In this study the term denaturation is meant to imply the formation of a product of lower solubility than that of the native protein.

respect to chemical properties, and second, to obtain evidence as to the nature of the energy transfer underlying the reactions.

Sonic Apparatus—Two types of apparatus were used to produce the sonic vibrations. One was a modified electromagnetic oscillator of a type employed in submarine communication and depth finding. The vibrating element is a stainless steel diaphragm about 30 cm. in diameter and 18 mm. thick. Such a unit may produce approximately 175 watts of acoustic energy when operated at the resonant frequency of 1200 cycles per second. Use of the diaphragm as the bottom of a cylindrical metal container made possible the treatment of from 1 to 5 liters of solution in direct contact with the vibrating surface. This electromagnetic oscillator was used only in experiments designed to determine the effect of different frequencies on the reaction.

The apparatus more extensively employed was the modified magnetostriction oscillator described by Chambers and Gaines (4). It consists of a cold-drawn, unannealed nickel tube, vibrating in a strong magnetic field in resonance with a 2000 volt oscillating power circuit to which the tube imparts approximately its own natural frequency. The vibrator used in these experiments was of such length that a frequency of about 9000 cycles per second was produced.

Two types of accessory apparatus were used in the experiments. A heavy aluminum pressure vessel designed by one of us and described elsewhere (5) was used for treatment of solutions under pressure, while the glass vessel shown in Fig. 1 was used for exposures under a vacuum and in the presence of gases other than air.

Materials—Egg albumin was prepared from eggs less than 24 hours old according to a modified Hopkins method (6). The product was purified by four recrystallizations and was then well dialyzed and diluted to 1 per cent (Kjeldahl). Solutions of Merck's egg albumin were used for comparative purposes, but no qualitative difference in type of reaction was observed.

Native amorphous horse serum albumin was prepared by the technique of Anson and Mirsky (7). We did not crystallize the material since Svedberg and Sjögren (8) have shown that repeated recrystallization of serum albumin gives an increasingly heterogeneous mixture. The solution was diluted to 2 per cent and 100 ml. were acidified with 30 ml. of 0.1 N HCl. Such an acidified

solution will flocculate, if, after heating in a boiling water bath, the pH be raised again with 0.1 N NaOH to the isoelectric region.

Plastein was prepared by the technique of Wastenays and Borsook (9), by using pepsin in a concentrated peptic digest of egg albumin at pH 4. The solid plastein was then separated

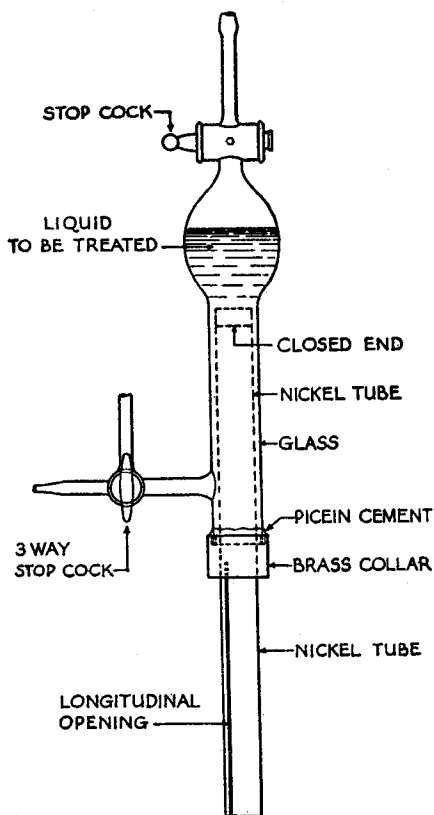


FIG. 1. Vacuum vessel

and well washed with water by centrifuging, and a solution of the material was prepared with 0.025 N HCl to a concentration of 1 per cent (Kjeldahl).

EXPERIMENTAL

When the 1 per cent egg albumin solution at the isoelectric point was vibrated in an open vessel, a white coagulum was

formed. In a 25 ml. sample approximately 20 per cent of the protein was so denatured in 4 minutes, although the amount varied from time to time owing to variations in acoustic output of the oscillator. This coagulum is soluble in dilute acid or alkali only to the extent of about 0.03 per cent (2). No part of the denaturation may be attributed to mass temperature rise in the solution, since adequate water cooling was provided to prevent an increase in temperature of more than 2° or 3°.

Prolonged treatment continued the production of coagulum but at the same time dispersed it into an opalescent sol which could not be separated by centrifuging at ordinary speeds (100 × gravity). In our previous study (2) no difference in immunological specificity between the products of moderately prolonged and short treatments was found; each gave reactions similar to those obtained with heat-denatured egg albumin.

Very prolonged sonic treatment (6 hours) apparently results in a further change in the coagulum (2), but we have not concerned ourselves with the nature of that end-product in this communication.

An investigation of the influence of the hydrogen ion concentration on sonic denaturation seems to corroborate the immunological finding that we are dealing with a thermal type of denaturation in the case of egg albumin. No coagulum was formed by 4 minutes vibration of the solution adjusted to pH 7.2 by addition of 0.15 N NaOH. (For some reason, unexplained as yet, the pH value fell to 6.7.) However, a shift in pH to the isoelectric point by adjustment with 0.025 N HCl subsequent to sonic treatment resulted in flocculation of 13 per cent of the original protein. Furthermore, the flocculated product was found to dissolve readily in excess of either acid or alkali. All of these peculiarities with respect to the conditions of pH parallel exactly those encountered when heat is the denaturing agent. Similarly, an acid solution of plastein was coagulated by sonic vibration under the pH conditions required for its coagulation by heat.

However, no such parallelism was observed in the case of horse serum albumin. Heat caused coagulation of a solution of this protein at pH 6, but no change was observed even by prolonged sonic treatment at this pH. Horse serum albumin vibrated at lower pH did not flocculate upon subsequent addition of 0.1 N

NaOH, although flocculation proceeded normally in a control portion when heating was substituted for the mechanical vibration. The chemical behavior of horse serum albumin differs from that of egg albumin in certain other respects. For example, the reversal of the denaturation of horse serum albumin is easily accomplished, but the reversal of denatured egg albumin has not been possible up to the present time (7, 10). Whether there is a relation between those proteins the heat denaturation of which is not reversible and those which are susceptible to sonic denaturation remains to be determined.

Although there is no reason thus far to doubt that the end-products of sonic and thermal denaturation are the same, when and if such products are formed, nevertheless the experiments give evidence that the mechanism of sonic denaturation does not parallel that of heat denaturation, as the studies on egg albumin alone seemed to indicate. Furthermore, the failure of horse serum albumin to be denatured indicates that the denaturations are not caused by possible momentary, localized temperature increases in the liquid resulting from adiabatic compressions, or from cavitation collapse. We have, therefore, undertaken a study designed to shed light on the mechanism through which sound waves energize the reaction.

When a liquid is exposed to the action of a strongly oscillating diaphragm or piston cavitation occurs; that is, evanescent vacuoles appear in the body of the fluid and at the surface of the vibrating element. The formation of these low pressure spaces has been discussed in detail by Gaines (11), and others, and practically all biological and chemical effects of intense sound heretofore described have been attributed either to the formation or collapse of the cavities. Since cavitation is the most obvious visible manifestation of sonic action on liquids, experiments were carried out to determine what relation exists between the denaturation reaction and the formation of the characteristic, vigorously active, gaseous or vapor bullæ. At the acoustic intensities available in the present study cavitation is produced in water with or without the presence of a dissolved gas, and only when an external pressure of approximately 6 atmospheres is applied to the liquid is cavitation completely inhibited.

On the other hand, the ultrasound source used by Wu and Liu

was reported by them to cause "gas bubbles" (cavitation) at atmospheric pressure in the presence of some gases (air, O₂, and H₂) but not in the presence of certain others (CO₂ and H₂S). Furthermore, no cavitation was caused in gas-free water (3). Wu and Liu found that egg albumin is coagulated by ultrasonic treatment under those conditions which allowed cavitation, and that no change is caused when the solutions fail to cavitate. From this observation they conclude that coagulation is brought about by condensation at the surfaces of the "gas bubbles," a reaction which can also be caused by vigorous manual shaking of the albumin solution in air.

A surface condensation mechanism, however, would not adequately explain the production in acid solution of a denatured product that is insoluble only at the isoelectric point. While our studies in the audible frequency range demonstrate that cavitation is essential to the reaction, further evidence made available by the possibility of producing cavitation in gas-free water and in water saturated with any type of gas, together with certain other observations recorded below, render improbable the conclusion of Wu and Liu regarding condensation at gas surfaces. It is true that coagulation of isoelectric egg albumin was not produced even by prolonged vibrations when cavitation in an air-saturated solution was completely inhibited by imposition of a hydrostatic pressure of 100 pounds per square inch. It is therefore evident that the denaturation occurs as a result of, or simultaneously with cavitation. However, one may not conclude from this fact that the sonic coagulation process is a surface phenomenon. Coagulation by shaking the solution with air may be inhibited by the addition of a surface-active substance such as saponin, but addition of saponin to solutions of egg albumin does not prevent coagulation by sound at normal pressure to any measurable degree.

Furthermore, sonic denaturation is not produced under all circumstances even when adequate cavitation is present. Although a gas-free solution of egg albumin literally boils during vibration under a vacuum, no coagulation results upon such treatment at the isoelectric point. Nevertheless, it seems definitely established that cavitation of the protein solution must occur in order that denaturation may take place.

In our experiments within the audible frequency range a specificity of the reaction toward certain gases was observed even though the acoustic intensity was such that cavitation was vigorous with all types of gases. Thus with air, oxygen, or carbon dioxide as the dissolved gas, coagulation of egg albumin solution treated at the isoelectric point reaches a maximum rate when pressure conditions allow ample cavitation; *i.e.*, at atmospheric pressure. On the other hand, with nitrogen or hydrogen coagulation was very nearly, if not completely, prevented.² The reaction was certainly very much more rapid when cavitation was ample in the presence of O₂ or CO₂ than was the case either with diminished cavitation, or with vigorous cavitation in the absence of dissolved gas or in the presence of hydrogen or nitrogen. The negative results with these two gases constitute evidence that sonic coagulation is not a surface condensation due to shaking.

We are dealing, then, with a reaction which requires, first, a sufficiently dense acoustic field to produce vigorous cavitation, and second, the presence of certain gases, *viz.* O₂ or CO₂. The implication seems clear that the denaturation is not a result of direct absorption of sound energy by the protein molecule, but is rather the result of an energy transfer through intermediary activated gas molecules. Furthermore, one may safely assume that the sonic activation of O₂ (demonstrated by Flosdorf, Chambers, and Malisoff (12)) and CO₂ in this sense takes place only under conditions favoring cavitation.

Such an energy transfer takes no account of chemical interactions between the activated gases or their products and the albumin molecule. This possibility should not be ignored. It is, however, difficult to reconcile the observation that both CO₂ and O₂ plus acoustic energy result in identical denatured end-products with any theory involving chemical combination. In the case of O₂ it has been shown by Flosdorf, Chambers, and Malisoff (12) that hydrogen peroxide is formed as a result of sonic activation in aqueous solution. The possibility of dena-

² It was difficult to determine whether or not there was complete absence of change, since there was always a trace of coagulated material present in the solutions as a result of the necessary preliminary manipulations. The detection of minute amounts of sonically produced coagulum was therefore impossible.

turation by peroxide was therefore checked by hand shaking and by sonic treatment of albumin solutions with H_2O_2 in concentrations of the order known to be present after 4 minutes vibration. No increased denaturation resulted. It appears then that the gas functions only as a carrier of energy to the protein molecule. That the transfer can be effected by certain gases and not by others under the same conditions may be correlated with the observations of Knudsen and Kneser (13) and of Kneser (14) that CO_2 and O_2 absorb far more acoustic energy at audible frequencies than does N_2 or H_2 . We realize that a hypothesis based on similarities between measurements made on materials in the gaseous state in the one case, and in a state of solution in the other, are precarious and at best can leave the specificity of the gases only inadequately explained. We are, nevertheless, impressed by the fact that of the four gases investigated the two gases showing maximum absorption and the two showing reaction specificity in our experiments are the same.

The wide differences in specificity toward N_2 and CO_2 represented by our findings at audible frequencies and by those of Wu and Liu at ultrasonic frequencies suggest that extensive investigation over a wide range of frequencies will be necessary to account for the discrepancies.

We wish to express our appreciation to Dr. Stuart Mudd for his helpful criticisms in this work.

SUMMARY

Egg albumin and plastein are denatured by intense sonic vibration. The solubility of the products under various conditions of pH is the same as that of the heat-denatured products.

Horse serum albumin is not denatured by sonic vibration. This fact suggests that the mechanism of the sonic reaction may differ from simple thermal activation.

Denaturation of egg albumin occurs only at acoustic intensities sufficient to promote vigorous cavitation of the solution. However, even at these intensities there is no denaturation when cavitation is suppressed by pressure or when cavitation is ample in the absence of dissolved gas.

The reaction proceeds actively when air, CO_2 , or O_2 is present,

but not in an atmosphere of either N_2 or H_2 , or in gas-free solution under a vacuum, even when the solution is vigorously cavitated.

A possible mechanism of sonic denaturation is discussed. The hypothesis requires direct transfer of energy from activated gas to protein molecules without chemical interaction.

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