THE INFLUENCE OF ULTRASONIC VIBRATIONS UPON THE ACTIVITY OF PARAMECIN*

by

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INTRODUCTION

For the purpose of disintegrating bacterial cells there have been developed several techniques which are adaptable to the disruption of Protozoa. These are based upon different principles ranging from autolytic rupture of the cell to grinding with powdered glass[2-7]. Although each method has merit for some specific problem, it frequently proves inadequate for other applications. Probably the greatest disadvantage of most of these methods is the addition of extraneous materials to the sample in order to effect the disintegration. A method completely avoiding this disadvantage is the use of ultrasonic vibrations.

STUMPF *et al.*[8] have described the application of ultrasonic disintegration to the preparation of cell-free enzyme extracts of bacteria. It was found that certain bacterial organisms were refractory to ultrasonic disintegration while others were easily disintegrated. Several factors affecting the efficiency of ultrasonic disintegration were found to be: the time of exposure, type of treatment container, concentration of the suspension, distance of the container from the crystal, and the effect of power input on the denaturation and inactivation of labile proteins. The only enzyme which was studied was pyruvic oxidase. The enzyme was found to be unaffected by the method of disintegration. HAAS[9] has reported the isolation of a soluble cytochrome oxidase preparation by a combination of mechanical disintegration with autolysis and ultrasonic vibration. Not all enzymes are, however, stable under the action of ultrasonic waves. BRABER *et al.*[10] found that ultrasonic waves (frequency of 960 kc/sec; 76 watts intensity) very rapidly inactivate aqueous solutions of the purified polyphenoloxidase of *Agaricus campestris*, in the presence of air. Ultrasonic disintegration has also been used successfully for the release of endo-enzymes from *Clostridium histolyticum*[11, 12] and the disintegration of bull sperm[13].

CHAMBERS AND FLOSDORF[14] using audible frequencies, and WU AND LIU[15] using ultrasonic frequencies, have demonstrated the denaturation of egg albumin in aqueous

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solution. CHAMBERS[16] investigated the effect of sonic vibration (9 kc/sec) on the proteolytic action of various preparations of pepsin. Recrystallized pepsin in acid solution was inactivated when exposed to these sound waves. It was found that certain unpurified and acidified pepsin preparations showed a more or less marked increase in proteolytic activity during the first few minutes of exposure to the vibrations. This activation process was found to occur under all conditions which allowed free cavitation in the sample. In air, a maximum activity was attained after which inactivation began. Preparations which were degassed and vibrated under pure nitrogen or hydrogen, resulted in a greater maximum activity after which there was no further change for the remainder of the treatment (20 minutes). The increased activity of the unpurified pepsin was tentatively attributed to increased availability of the enzyme caused by sonic dispersal of molecular aggregates.

The effect of ultrasonic vibrations on macromolecular substances has been investigated to a limited extent. SZENT-GYÖRGI demonstrated that starch was decomposed by ultrasonic waves to achrodextrins[17]. Decomposition of the molecules of highly polymerized substances was observed by SZALAY[18], who suggested that the action of ultrasonic waves may be other than mechanical. Only in the case of very large molecules, such as those of hemocyanin, did a breakdown of molecules by ultrasonic vibrations seem possible from purely mechanical considerations. According to BROHULT[19] the molecules of hemocyanin are disrupted by ultrasonic waves to molecules which have only $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, and $\frac{1}{16}$ of their original size. Similar results were obtained by SOZABURO[20]. SCHMIDT[21–23] found that the molecular weight of polystyrenes dissolved in toluene decreased under the influence of ultrasonic waves and explains the breakdown of the macromolecules by the difference in the deformability between these molecules and the solvents.

LEPESCHKIN[24] using ultrasonic waves with a frequency of 285 kc found an increase in the average molecular weight of serum proteins if the reaction of the serum was alkaline and the exposure was short (about 1 minute). Longer exposures resulted in decreased weight. In acid solution only a decrease in molecular wight was observed. In previous work[25] LEPESCHKIN had concluded that salt-like inner bindings of protein molecules can be destroyed by purely mechanical action of the displacement current in a high-frequency electromagnetic field. Ultrasonic vibrations were supposed to exert a similar effect on serum proteins. The salt-like inner bindings of protein molecules would be destroyed when the molecular weight decreased or formed if it increased. The denaturation of horse serum albumin, pseudoglobulin, and euglobulin, upon subjection to ultrasonic vibrations of 960 kc frequency, has been reported by PRUDHOMME AND GRABAR[26].

TAKAHASHI AND CHRISTENSEN[27] found that the juice of plants suffering from tobacco mosaic virus disease was rendered non-infectious when subjected to sonic vibration. STANLEY[28] reported that the biological activity of purified tobacco mosaic virus was reduced by sonic treatment and demonstrated that the sound waves had little or no effect on the activity of the virus if cavitation, normally associated with strong vibrations in liquids, is suppressed by a lowering of the atmospheric pressure above the liquid. This is in agreement with CHAMBERS[16] who found that inactivation of pepsin did not occur in degassed samples. KAUSCHE, PFANKUCH, AND RUSKA[29] demonstrated that ultrasonic treatment apparently results in a breakage into shorter fragments of the long rod-like particles of tobacco mosaic virus. OSTER[30] confirmed this observation

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and extended it by determining the correlation between size distribution and biological activity by means of electron microscope studies. The biological activity of tobacco mosaic virus, as determined by the half-leaf lesion method, decreased exponentially with the time of ultrasonic treatment. A correlation was found to exist between the size distributions and the biological activity: only the virus particles 280 millimicrons in length are biologically active.

The degree of resistance of Tubercle bacilli to ultrasonic disintegration has been studied[31] and a mechanism proposed for the mode of disruption[32, 33]. The disintegration of *Paramecium caudatum* by intense sound waves of audible frequencies has also been studied[34].

Paramecin, the "killer" substance of *Paramecium aurelia* (stock 51, variety 4) is probably a desoxyribonucleoprotein[35] which is easily inactivated under mild conditions[36, 37]. In view of the above considerations, it was desirable to determine the feasibility of using high-frequency ultrasonic vibrations as a method of disintegrating large numbers of paramecia as a preliminary step in the isolation of paramecin. After determining whether paramecia can be successfully disintegrated in this manner it becomes essential to determine the effect of ultrasonic vibrations on paramecin. By extending these determinations to include ultrasonic procedures which give characteristic changes in proteins, it seemed possible to obtain corroborative evidence concerning the protein nature of paramecin.

METHODS AND RESULTS

Detailed descriptions of the method of cultivating *Paramecium aurelia*[38] and of the procedure for testing the activity of the paramecin breis have been published[36]. The ultrasonic instrument* used has a maximum electrical input of *c.* 3000 volts and 350 ma. giving approximately 1000 watts as the maximum power input. The generator operates at 85% efficiency, thereby applying *c.* 850 watts at the crystal. The quartz crystal holder is immersed in oil. On the basis of a radiation efficiency of 10 watts per square centimeter[39], the vibrational or sound energy at the crystal exceeds 300 acoustical watts. The quartz crystal has a fixed frequency of 450,000 cycles per second; the amplitude of the ultrasonic waves and thereby the intensity, is controlled by variation of the generator input. Temperature regulation was obtained by circulating the oil from the bath through copper coils immersed in a dry-ice ether mixture contained in a large Dewar flask. In this manner the bath temperature could be maintained at approximately 6° C during the course of the experiments.

Two types of treatment containers were used: for small samples a test tube (25 mm × 200 mm) with the bottom blown out to a thin flat membrane was utilized; for large samples, the container consisted of a large copper or stainless steel tube (8 cm in diameter) with a thin (*c.* 0.03 mm) copper or stainless steel diaphragm sweated across the bottom. Glass containers have an inherent disadvantage in that a large loss in energy (up to 70%) occurs whereas in the case of the metal diaphragms only about a 10% loss occurs. The large container has the advantage that a cooling coil can be placed directly in the sample.

Disintegration of whole paramecia

Experiments were carried out to determine the feasibility of using high-frequency ultrasonic vibrations to disintegrate large numbers of *Paramecium aurelia* (stock 51, variety 4) as a preliminary step in the isolation of paramecin. *Paramecia* were concentrated by means of filtration with a Berkefeld filter and subsequent centrifugation[40]. The whole animals were resuspended in phosphate buffer to a final concentration of approximately 5000 animals per ml. The p_H of the phosphate buffer was 8.0, at which p_H paramecin is most stable[36]. Using the glass treatment container, complete disintegration was not achieved with maximum input of the instrument for 5 minutes. Para-

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mecin was completely inactivated during this treatment. Complete disintegration could be obtained by using the metallic diaphragm container and vibrating at maximum input for 5 minutes but was accompanied by losses in paramecin activity ranging from 50–100%. The discrepancy in inactivation in the two containers can be ascribed to heat inactivation in the glass container. Due to the relative inelasticity of the glass membrane, a large amount of the ultrasonic energy is dissipated as heat. The energy necessary to obtain complete disruption of the paramecia was too great to maintain an appreciable paramecin activity in the obtained brei.

Effect of ultrasonic vibrations on paramecin activity

For the investigation of the effect of ultrasonic vibrations on paramecin activity, paramecium breis were used exclusively. These breis were prepared by repeatedly forcing a concentrated suspension of killer paramecia through a fine gauge (No. 27) injection needle until complete disintegration of the whole animals was obtained[40]. All the experiments were performed with the glass treatment container. The temperature of the ultrasonic bath (6° C) did not vary significantly for the time intervals and inputs used.

Inactivation of paramecin at the temperature of the ultrasonic bath

A control curve for the inactivation of paramecin at the temperature of the ultrasonic bath was required as a basis for determining the effects actually due to ultrasonic vibrations. The brei was placed in the treatment container, immersed in the ultrasonic bath, and samples were taken at the same time intervals as those in the actual treatments with vibration. The average of the results from three experiments are given in Table I and demonstrate that there is no significant change in activity due to the low temperature over the period of ultrasonic treatment.

TABLE I

THE INACTIVATION OF PARAMECIN AT THE TEMPERATURE OF THE ULTRASONIC BATH

Time in Minutes	0.0	0.5	1.0	2.0	3.0	5.0	10.0	15.0
Activity in %	100	99	100	98	100	100	100	101

Determination of the maximum effects of vibration of the activity of paramecin breis

In order to determine the effect of ultrasonic vibrations on the activity of paramecin over the whole amplitude range of the instrument, an arbitrary time of exposure of 0.5 minutes was chosen. Exposure of breis to 0, 40, 174, 405, and 613 watts electrical input for 0.5 minutes indicated somewhat increased activity at 40 watts input and decreased activities at 613 watts (Table II) input.

TABLE II

EFFECT OF ULTRASONIC VIBRATIONS AT DIFFERENT INTENSITIES AND CONSTANT TIME OF EXPOSURE ON THE ACTIVITY OF PARAMECIN

Inputs in Watts	0	40	174	405	613
Activity in %	100	118	100	90	73

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The ultrasonic intensity at which the greatest increase in paramecin activity occurs was more accurately determined by using smaller increments of electrical input. Aliquots of an originally large brei were exposed for 0.5 minute intervals at steps of 20 ma. from 0–200 ma. The results of this experiment are given in Fig. 1. The maximum increase in activity is seen to occur at 20 watts. This increase is reproducible although the actual increase in activity varies more or less in different samples.

The effect of varying the time of exposure at 20 watts input on the activity of paramecin are given in Fig. 2.

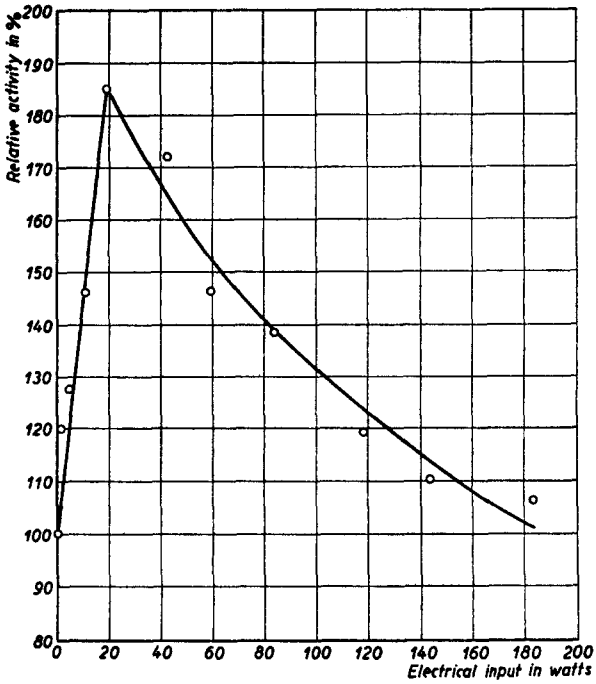

Fig. 1. Effect of varying intensities of ultrasonic treatment on the activity of paramecin with a constant time of exposure (0.5 min)

Effect of oxygen and nitrogen on the activity of paramecin during ultrasonic vibrations

The presence of oxygen or nitrogen exerts an effect on the action of ultrasonic vibrations. It was therefore of interest to determine whether these gases influenced the mode of inactivation of paramecin breis when exposed to ultrasonic vibrations. This necessitated a preliminary degassing of the samples.

a. *Method of degassing samples.* Although it is possible to degas samples directly in an instrument of the magnetostriction type[16], such a procedure is not applicable to the containers used for a quartz crystal instrument. In order to carry out this step, the simple auxiliary apparatus illustrated in Fig. 3 was constructed. The brei was placed in chamber D and subjected to a vacuum of 10–20 microns by opening stopcock A. Stem E extends to the bottom of the treatment container, which is swept free of air by means of the gas (nitrogen or oxygen) which is being used for the particular experiment.

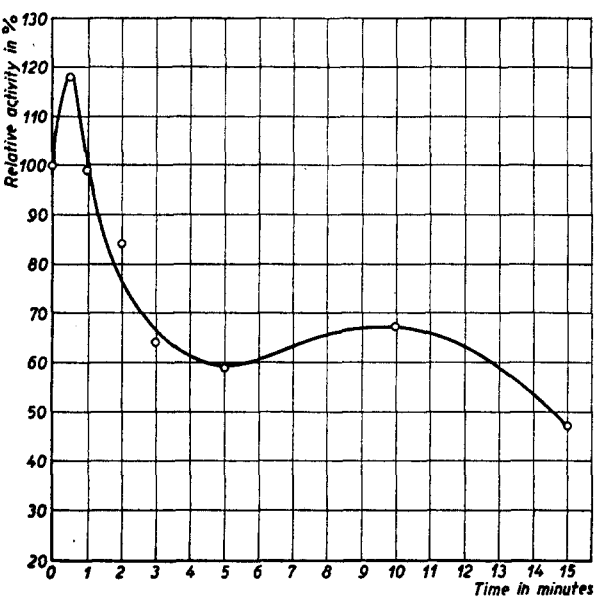

Fig. 2. Effect of ultrasonic vibrations on the activity of paramecin with a constant input of 20 watts over varying periods of time under an air atmosphere (sample not degassed)

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By barely opening stopcock C, a stream of gas may be used to clear the stem of air. After approximately 10 minutes under vacuum, stopcock A is closed and the sample allowed to drain into the treatment container by opening both stopcocks B and C. The brei is then kept under an atmosphere of the appropriate gas by a jet placed just over the surface.

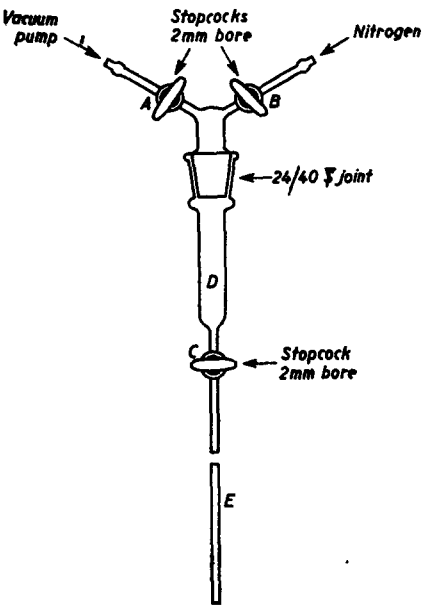

Fig. 3. Schematic diagram of auxiliary apparatus for degassing samples for ultrasonic vibration

b. *Effect of oxygen upon the ultrasonic vibration of paramecin.* Two types of experiments were carried out in which vibration of the brei took place under an oxygen atmosphere: in one, the oxygen jet was placed over the surface of the brei and in the other, the oxygen was bubbled directly through the brei. In both cases the breis were degassed by the method described and subjected to ultrasonic vibrations (at 20 watts electrical input). Samples were taken at specified time intervals. The results for both experiments are given in Fig. 4. The increase in activity, observed in the vibration of a non-degassed brei in the presence of air, is seen to be very slight when the oxygen jet is placed over the surface of the sample and completely absent when the oxygen is bubbled directly through the brei. In the latter case, the inactivation curve during the first 5 minutes approximates that of a first order reaction. The secondary increase in activity, which occurs in 10 minutes, is seen to take place in both the presence of air and when oxygen is bubbled directly into the brei but is almost completely absent when the oxygen atmosphere is maintained by placing the oxygen jet over the brei.

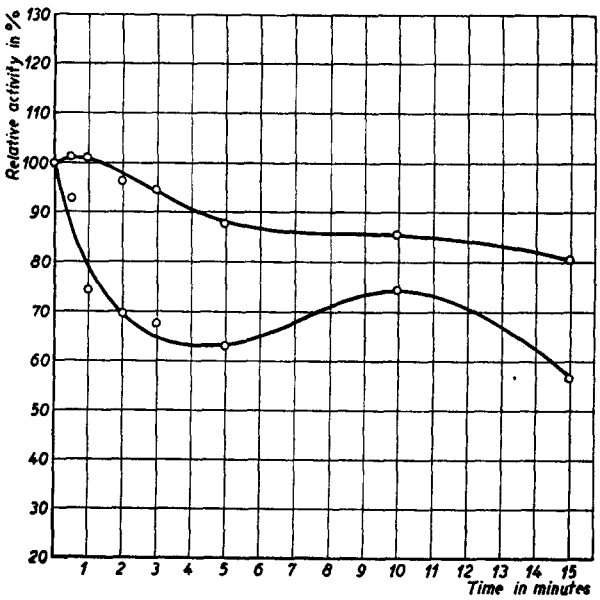

Fig. 4. Effect of ultrasonic vibrations on the activity of paramecin with a constant input of 20 watts over varying periods of time with an oxygen atmosphere. The upper curve represents the change in activity when the brei is vibrated under an oxygen atmosphere and the lower curve represents the change in activity when vibration takes place with oxygen bubbling directly through the sample. Samples degassed before vibration.

c. *Effect of nitrogen upon ultrasonic vibration of paramecin.* Different results are obtained when ultrasonic vibration is carried out in a nitrogen atmosphere (maintained with the nitrogen jet over the surface of the brei). The results are given in Fig. 5. After an initial small increase in activity, a drop to about 95% of the original activity occurs with no further change for the duration of the experiment.

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Centrifugation of paramecin after ultrasonic treatment of a paramecin brei

Paramecin has been found to be easily sedimented by relatively low centrifugal force[37, 41]. Since several enzyme systems could be separated by ultrasonation[8, 9] from the cellular constituents to which they were bound, a similar treatment might bring paramecin into solution. Experiments designed to test this possibility are described below.

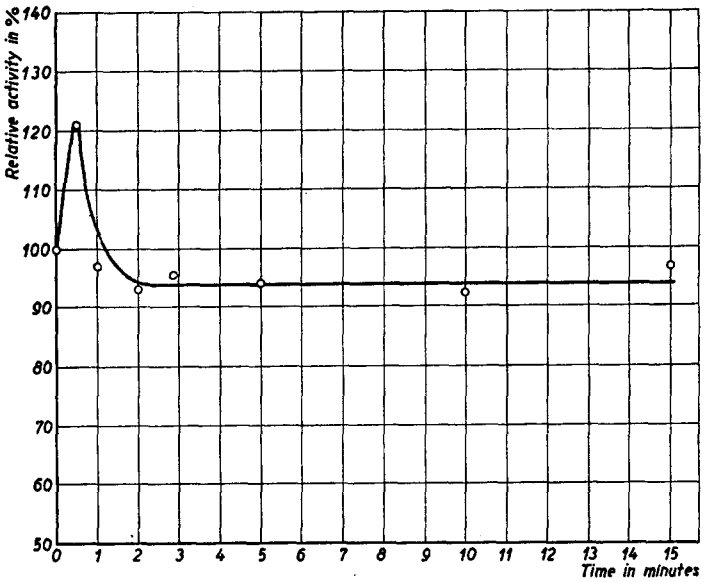

Fig. 5. Effect of ultrasonic vibrations on the activity of paramecin with a constant input of 20 watts over varying periods of time under a nitrogen atmosphere. Samples degassed before vibration

Brei samples were subjected to high-intensity ultrasonation for different lengths of time. The samples were subsequently centrifuged as indicated in Fig. 6, and the activities of the supernatants and sediments determined. Sample #1 represents the control sample and was kept at the same temperature as that of the ultrasonic bath (6° C). Decreased activity

5 samples, each containing ca. 248,000 Paramecium aurelia 51, variety 4 in 15 cc. of phosphate buffer (p_H 8.0), made into breis by the syringe method.

Sample #1	Sample #2	Sample #3	Sample #4	Sample #5
675,000 Pn	642,000 Pn	465,000 Pn	870,000 Pn	634,000 Pn
		Ultrasonated at 525 watts for time given		
0 min	0.5 min	1.0 min	2.0 min	10.0 min
Sample #1	Sample #2	Sample #3	Sample #4	Sample #5
757,000 Pn	375,000 Pn	304,000 Pn	100,000 Pn	18,000 Pn

5 ml of each sample centrifuged 15 minutes at 400 × gravity in type A Sorval angle centrifuge. Sediments resuspended to 5 ml in original phosphate buffer (p_H 8.0).

Supernatant	Supernatant	Supernatant	Supernatant	Supernatant
3740 Pn	11,200 Pn	7500 Pn	0 Pn	0 Pn
Sediment	Sediment	Sediment	Sediment	Sediment
1,020,000 Pn	395,000 Pn	268,000 Pn	113,000 Pn	7500 Pn

Fig. 6. Centrifugation of Paramecin Breis after Ultrasonation

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with increased time of exposure to high-intensity ultrasonic vibrations is apparent in this experiment. The increases in activity which occur in samples #1, 2, and 4 have been observed before under other conditions[36, 37].

The small amounts of activity remaining in the supernatants of samples #1–3 were not due to dissolved paramecin. Duplication of these experiments with 30 minutes centrifugation at 400 × G instead of 15 minutes results in complete sedimentation of paramecin.

DISCUSSION OF RESULTS

The results obtained in these experiments are similar in several respects to those obtained by CHAMBERS[16] on the activation and inactivation of impure pepsin preparations. With a non-degassed sample under an air atmosphere, the primary increase in activity and subsequent inactivation (up to 5 minutes) of paramecin by ultrasonic vibrations parallels the same phenomenon with impure pepsin. However, the secondary increase in activity presented by paramecin breis when vibrated under atmospheres of air or oxygen (directly into sample) is not shown by pepsin. This delayed activation response, which is observed in 10 minutes with the method used, cannot be explained at present. This same phenomenon of secondary activation has been observed during temperature inactivation experiments in various salt solutions in which the secondary increase in paramecin activity occurred in 40 minutes[37].

The variation in response obtained with atmospheres of air and oxygen is subject to partial explanation. A degassed sample, vibrated under oxygen, does not contain initial dissolved oxygen which could contribute to either an activation or an inactivation. As the ultrasonic vibration proceeds, oxygen is dissolved and an inactivation appears to be the governing reaction. When a brei is vibrated under air without degassing, the reaction is first one of activation followed by an inactivation and then a secondary activation. This secondary activation, which also appears when oxygen is bubbled through the brei, would seem to indicate that a certain minimal amount of dissolved oxygen is necessary for this reaction. It is also conceivable that this secondary activation is completely independent of the primary one.

Increases in paramecin activity have been reported for crude paramecin preparations in phosphate buffer (p_H 8.0) at 20° C[36]. This increase was spontaneous and probably of the same origin as that observed in the ultrasonic experiments. Although the mechanisms for activation and inactivation of paramecin, either spontaneously or by ultrasonic treatment, are unknown, several possibilities may be considered for these reactions. Increases in the activity of a crude paramecin brei may be due to degradation of a high-molecular aggregate which is the precursor of paramecin in the cell, to reactivation of degraded paramecin, to a deaggregation of particles containing one unit of paramecin whose activity has been masked by the aggregate, or to deaggregation of aggregates containing two or more units of paramecin. Paramecin breis which have been subjected to ultrasonic treatment followed by centrifugation have shown that the whole of the activity is found in the sediment and none in the supernatant thereby precluding the possibility that increases in activity are due to increased availability of paramecin by the production of a soluble form. Inactivation of paramecin by ultrasonic vibrations can probably be attributed to denaturation of the protein component of paramecin since denaturation of proteins by ultrasonic vibrations is a characteristic reaction[10, 24, 26, 28, 29, 30].

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SUMMARY

1. Completely disintegrated suspensions of *Paramecia* may be obtained by subjection of live *Paramecia* suspensions to ultrasonic vibrations (450 kilocycles, 850 watts electrical input, metal diaphragm container). Paramecin is largely or wholly destroyed by this treatment.

2. Paramecin breis have been subjected to ultrasonic treatment under a variety of conditions. A maximum increase in activity of non-degassed samples is found to occur with an exposure of 0.5 minutes at 20 watts electrical input, when vibrated under an air atmosphere. A smaller secondary increase in activity occurred after 10 minutes ultrasonic treatment. When the brei is degassed and vibrated under an atmosphere of oxygen maintained with oxygen jet above sample there is only a very slight increase in activity during the first minute after which inactivation takes place. If oxygen is bubbled directly through the sample during vibration, inactivation commences immediately and continues for 5 minutes. This is then followed by an activation in 10 minutes and subsequent return to the inactivation reaction in 15 minutes. Samples degassed and vibrated under nitrogen gave an increase in activity in the first half-minute, followed by an inactivation to approximately 95% of the original activity in a minute and maintenance of this value for the remainder of the treatment.

3. Centrifugation of paramecin breis after ultrasonation demonstrates that a soluble form of paramecin is not produced by ultrasonic vibration.

RÉSUMÉ

1. Il est possible d'obtenir des suspensions complètement désintégrées de *Paramecia* en soumettant des suspensions de *Paramecia* vivantes à des vibrations ultrasoniques (450 kilocycles, 850 watts, cylindre à diaphragme métallique). Par ce traitement, la paramécine est largement ou complètement détruite.

2. Les traitements ultrasoniques de suspensions de paramécine ont été effectués dans différentes conditions. L'accroissement d'activité maximum d'échantillons non dégazés a été déterminé pour une exposition de 0.5 minutes, 20 watts dans l'air. Un petit accroissement secondaire d'activité es produit après un traitement ultrasonique d'une durée de 10 minutes. Quand la suspension est dégazée et traitée aux ultrasons dans une atmosphère d'oxygène, maintenue au-dessus de l'échantillon par un jet d'oxygène, l'accroissement d'activité durant la première minute est très faible après quoi une désactivation se produit. Si l'oxygène est bouillonné directement au travers de l'échantillon durant le traitement ultrasonique, la désactivation commence immédiatement et continue durant 5 minutes. Ceci est ensuite suivi par une activation après 10 minutes et un retour ultérieur de la désactivation après 15 minutes. Des échantillons dégazés et traités aux ultrasons dans l'azote donnèrent un accroissement d'activité durant la première demi-minute, suivi en une minute par une désactivation jusqu'à environ 95% de l'activité originale, cette valeur se maintenant ensuite pour le restant du traitement.

3. La centrifugation de suspensions de paramécine après le traitement ultrasonique prouve que ce dernier ne produit pas de forme soluble de paramécine.

ZUSAMMENFASSUNG

1. Suspensionen von vollkommen zertrümmerten Paramäcien werden erhalten, wenn man Suspensionen von lebenden Paramäcien Ultraschall-Wellen aussetzt (450 Kilozyklen, 850 Watt, Metallmembran-Behälter). Paramäcin wird durch diese Behandlung grössenteils oder vollkommen zerstört.

2. Paramäcienbrei wurde unter verschiedenen Bedingungen der Ultraschallbehandlung ausgesetzt. Wenn die Beschallung bei Luftzutritt geschieht, erfolgt der maximale Anstieg der Aktivität von nicht entgasten Suspensionen bei einer Behandlung mit 20 Watt während 0.5 Minuten. Ein zweiter geringerer Anstieg der Aktivität erfolgt nach 10 Minuten Ultraschallbehandlung. Wenn der Brei entgast wird und die Ultraschallbehandlung unter Einblasen von Sauerstoff oberhalb der Suspension erfolgt, so steigt die Aktivität während der ersten Minute ein wenig an; dann nimmt die Aktivität ab. Wenn Sauerstoff während der Beschallung direkt durch die Suspension geblasen wird, nimmt die Aktivität sofort ab und sinkt weiter während 5 Minuten. Darauf folgt Aktivierung während 10 Minuten and weiter Inaktivierung während der nächsten 15 Minuten. Suspensionen die entgast und in Stickstoffathmosphäre beschallt wurden, zeigten Anstieg der Aktivität in der ersten halben Minute, dann Abnahme der Aktivität um etwa 95% des ursprünglichen Wertes und Beibehaltung dieses Wertes während der weiteren Behandlung.

3. Zentrifugiert man die mit Ultraschall behandelten Suspensionen von Paramäcium, so erhält man kein lösliches Paramäcin in der überstehenden Flüssigkeit.

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