SONIC DISRUPTION OF *AZOTOBACTER VINELANDII*[1]

ALLEN G. MARR AND E. H. COTA-ROBLES[2]

Department of Bacteriology, University of California, Davis, California

Received for publication January 21, 1957

The exposure of microorganisms to intense sound has been used by many workers for the extraction of enzymes and antigens; however, the investigators have rarely reported sufficient quantitative data to permit a correlation of the disruption of cells with the release of the enzyme or antigen. Such information is particularly important in evaluating the distribution of enzymes in centrifugal fractions of disrupted bacterial cells. The submicroscopic particles in extracts of bacteria have been found to contain most of the ribonucleic acid (Schachman *et al.*, 1952) and many of the enzymes (Alexander, 1956). These submicroscopic particles may be actual cytological elements or artifacts resulting from the method of disrupting the cell. A correlation of the release of substances in the particulate fractions with other parameters of sonic disruption of the cells has permitted a decision between these two possibilities.

METHODS

Preparation of cell suspensions. Mass cultures of *Azotobacter vinelandii* strain O[3] (*A. agile* var. *vinelandii*) were grown in 9-L pyrex bottles containing 7 L of Burk's nitrogen free medium consisting of: K_2HPO_4, 0.8 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.05 g; $Na_2MoO_4 \cdot 2H_2O$, 0.00025 g; $FeNH_4(SO_4)_2 \cdot 12H_2O$, 0.0086 g and sucrose 20 g per L. The inoculum was a 75 ml culture in the same medium grown for 24 hr on a rotary shaker. The mass culture was aerated with a sparger consisting of a large alundum extraction thimble fitted with a glass inlet and glass-wool filter. The rate of aeration was 1 L per min for the first 6–8 hr, after which it was increased to 10 L per min. The cultures were incubated for 24–30 hr at 30 C and harvested by centrifugation. The cells were washed at least 3 times by centrifugation for 10 min at 2,000 × G from distilled water; this centrifugation packs the cells but permits the slime to be decanted. The washed cells were used immediately or stored at −10 C.

Viable cells were counted by spreading 0.10 ml aliquots of appropriate dilutions on plates of Burk's medium containing agar. Direct counts were made in a Spencer bright-line hemocytometer for phase microscopy with a Zeiss Ph 2 objective and a 10× compensating ocular. Turbidity was determined with a Klett-Summerson colorimeter using a red filter.

Sonic disruption. Forty ml of a suspension of cells in 0.05 M phosphate buffer at pH 6.8 containing 200 mg wet weight of cells per ml was added to the transducer of a 10 KC Raytheon sonic oscillator. The gas phase was replaced with H_2 by flushing for several minutes; this precaution is necessary to prevent oxidative inactivation of enzymes resulting from cavitation of water containing dissolved O_2 (Lindström, 1955). The suspension was exposed to a sound field of approximately 50 acoustical watts (1.2–1.3 amp audio frequency current) unless stated otherwise. The temperature was maintained below 3 C during treatment. Aliquots were removed and replaced with an equal volume of buffer.

Enzymatic assays. (1) Hydrogenase:—Assayed by measuring manometrically the gas consumed in the enzymatic reduction of methylene blue at 30 C (Gest, 1952). The vessels contained 0.50 ml of 0.016 M methylene blue, 0.50–1.00 ml of enzyme in buffer and 0.05 M phosphate buffer, pH 6.8, to make 3.00 ml. The center well contained 0.20 ml of 20 per cent KOH and the atmosphere was H_2. The enzyme was diluted, if necessary, to give a rate of 1 to 4 μL of H_2 consumed per minute.

(2) Glucose-6-phosphate dehydrogenase (G-6-PDH):—Assayed by measuring the rate of reduction of diphosphopyridine nucleotide (DPN) spectrophotometrically (Mortenson and Wilson, 1954). The samples were centrifuged for 2 hr at 100,000 × G to remove DPNH oxidase which interferes with the assay.

Chemical determinations. Cytochrome was estimated by measuring the optical density at

[1] Supported by a grant from the National Science Foundation.

[2] Present address: Division of Life Science, University of California, Riverside, California.

[3] Obtained from the collection of Dr. P. W. Wilson, University of Wisconsin.

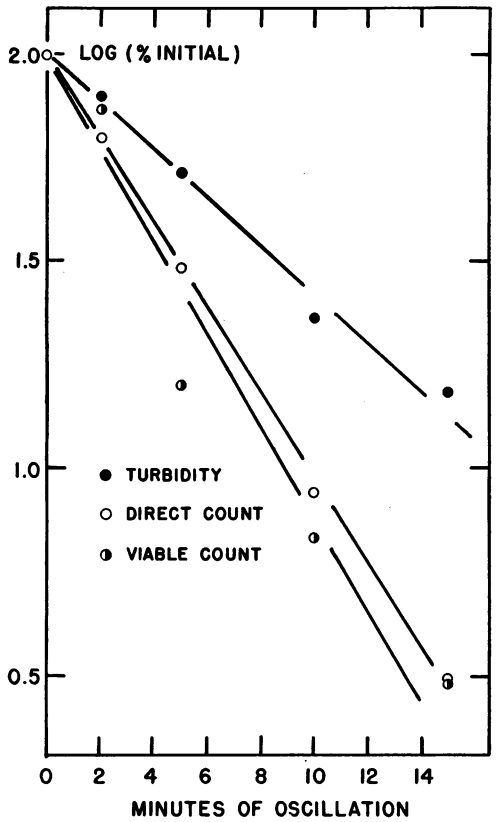

Figure 1. Turbidity, direct count and viable count as functions of the time of sonic treatment.

418 mμ, the absorption maximum of the Soret peak, and subtracting the optical density at 460 mμ to partially compensate for light scattering.

Phospholipid and nucleic acids were determined by the method of Schneider (1945). Samples were extracted with cold trichloracetic acid; the residue was then extracted with 95 per cent ethanol to obtain the phospholipid. Since boiling with ether-ethanol removed no additional phosphorus, this step was omitted. Nucleic acid was extracted with hot trichloracetic acid. Total nucleic acid was determined both by optical density at 260 mμ and by total phosphorus. Deoxyribonucleic acid (DNA) was determined by reaction with diphenylamine (Dische, 1930). Ribonucleic acid (RNA) was estimated by difference. Phosphorus was determined by the method of Allen (1940).

Kinetics of disruption. Direct count and turbidity were measured as parameters of the effect of sonic treatment. Figure 1 shows that the rates of change of both parameters are exponential; the logarithm of the fraction remaining is a linear function of the time of treatment. The rate of decrease in turbidity is only 40 per cent of the rate of decrease in number of cells. Thus, the decrease in turbidity does not result from the rupture of the cell *per se*. The agreement between direct and viable count is sufficient to show that the chief mechanism of killing is disruption of the cell.

Since the turbidity after treatment exceeds that attributable to residual intact cells, some subcellular structure which is large enough to scatter red light must accumulate. The material responsible for most of the turbidity can be removed by centrifugation at 2,000 × G for 10 min; this further suggests microscopic dimensions. Figure 2*a* is a phase photomicrograph of

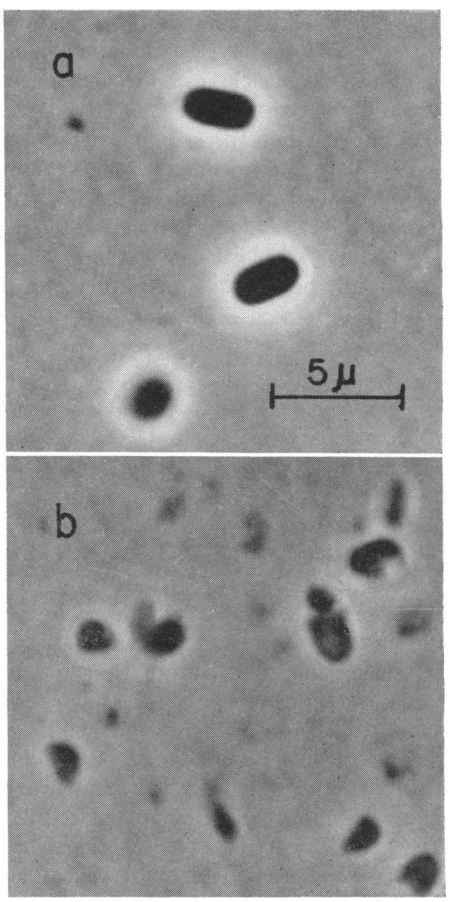

Figure 2. Phase photomicrographs of *Azotobacter vinelandii* (*a*) before treatment and (*b*) after sonic treatment for 1 min. The samples were mounted in 2 per cent agar and photographed on Adox KB-14 film with a Zeiss Ph 100 neofluar objective and 15X compensating ocular.

untreated cells of *A. vinelandii*, and figure 2*b* a preparation after brief sonic treatment. The contour and diminished contrast identify the structures as large fragments of the cell envelope emptied of its cytoplasm. These fragments which will be designated as *hulls* have been observed by others following sonic treatment (Hamre, 1949; Mudd and Lackman, 1941). The hull undoubtedly contains the cell wall and may contain an adhering membrane.

The accumulation of hulls during sonic treatment of *A. vinelandii* results in a rate of decrease in turbidity lower than the rate of disruption of cells. Since the decrease in turbidity is exponential even in the region in which residual intact cells contribute a negligible fraction of the turbidity, it must be assumed that turbidity is a function of the amount of cell envelope whether the envelope is an empty hull or surrounds an intact cell.

The rates of change of cells and turbidity can be expressed as follows:

Let C = the fraction of the initial cells
T = the fraction of the initial turbidity (or cell envelope)
k_c = the apparent rate constant for change in number of cells
k_y = the apparent rate constant for change in turbidity
t = time of treatment
e = the base of natural logarithms

Since the rate of change of both C and T were found to be exponential:

(1a) $C = e^{-k_c t}$ (1b) $T = e^{-k_y t}$

and

(2a) $-\frac{dC}{dt} = k_c C$ (2b) $-\frac{dT}{dt} = k_y T.$

Equations 1a and 1b could describe two distinct mechanisms: (a) Two separate reactions; the faster reaction (equation 1a) ruptures the cell and the slower reaction (equation 1b) fragments the residual envelopes to submicroscopic particles and decreases the turbidity. (b) A single reaction; a hit on a cell disintegrates a portion of the cell envelope which destroys the cell (a fractional cell is impossible). If equations 2a and 2b describe separate reactions, the ratio of the constants k_y/k_c might be expected to vary as the intensity of the sound field is decreased. Table 1 shows the results of an experiment in which the audio frequency current through the transducer was varied from 0.75 to 1.25 amperes, which increases the audio frequency power from 23 to 52 acoustical watts. Although both rate constants increase as the intensity of sound is increased, the ratio remains constant.

TABLE 1

The effect of the intensity of sound on the rate constants of turbidity and direct count

Amperes Audio Frequency	Acoustical Watts*	k_y†	k_c	k_y/k_c
0.75	23	0.093	0.25	0.37
1.10	42	0.35	0.15	0.43
1.25	52	0.27	0.69	0.39

* Measured by direct calorimetry.

† The symbols k_y and k_c are defined in equations 1a and 1b and have dimensions of minutes^{-1}.

The significance of the fixed ratio of the two rate constants can be established as follows:

Substituting from equations 1a and 1b into 2a and 2b,

(3a) $-\frac{dC}{dt} = k_c e^{-k_c t}$ (3b) $-\frac{dT}{dt} = k_y e^{-k_y t}.$

The change in turbidity with respect to change in number of cells is given by

(4) $$\frac{dT}{dC} = \frac{k_y e^{-k_y t}}{k_c e^{-k_c t}}.$$

At zero time, when only intact cells are contributing to turbidity, equation 4 becomes

(5) $$\frac{dT}{dC} = \frac{k_y}{k_c}.$$

Thus the ratio of the rate constants, k_y/k_c is the change in turbidity with respect to change in number of cells if only intact cells are contributing to turbidity. If turbidity is a measure of the amount of surface of cell envelope, this ratio is the fraction of the cell envelope disintegrated by a single hit on a cell. Since the ratio is approximately 0.4, an average hit disintegrates 40 per cent of the cell envelope, which agrees with estimates made by direct microscopic observation after brief sonic treatment.

Kinetics of release of substances from the cell. The rate of release of a substance from the cell might reveal, in part, the location of that substance in the cell. Substances which are not readily sedimented by centrifuging cell extracts, the so-called soluble fraction, are generally assumed to exist as such in the cytoplasm rather than to be derived

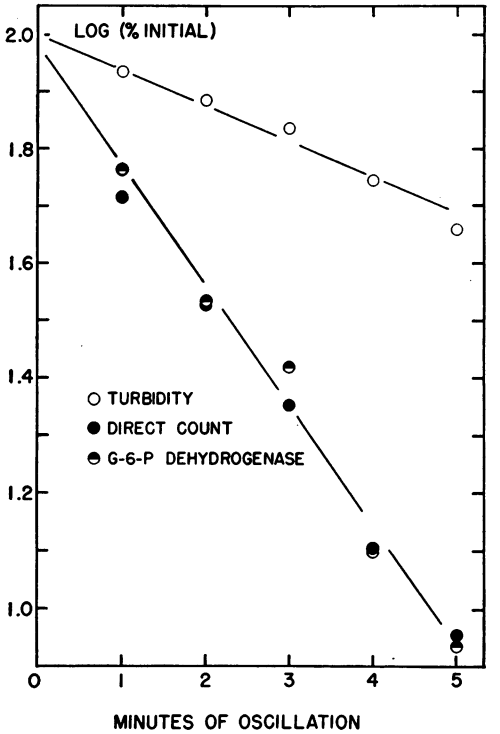

Figure 3. The rate of release of glucose-6-phosphate dehydrogenase by sonic treatment. The enzyme was assayed in the supernatant liquid after centrifuging at 2,000 × G for 10 min and the amount sedimented calculated by subtracting from the total enzyme released. The logarithm of the per cent of the total enzyme which is sedimented by centrifugation is the function plotted.

by the destruction of a larger, more integrated structure. If such an assumption is correct, soluble substances should be released at a rate identical with the rate of disruption of cells.

Glucose-6-phosphate dehydrogenase (G-6-PDH) has been found in the soluble fraction of cell free extracts of *A. vinelandii* (Cota-Robles, 1956). A comparison of the rate of release of this enzyme with the turbidity and direct count is shown in figure 3. The enzyme was assayed in the supernatant after centrifuging the samples at 2,000 × G for 10 min which is sufficient to remove residual cells and hulls. The activity released after 5 min sonic treatment was taken as the total; the amount of enzyme able to be sedimented was determined by subtracting the amount in the supernatant from the total. G-6-PDH is released at the same rate as the decrease in direct count. Thus, when a cell is disrupted, its entire content of this enzyme is released.

The particulate fraction of cell free extracts is arbitrarily defined as submicroscopic particles sedimenting in 2 hr or less at 100,000 × G. This fraction of extracts of *A. vinelandii* contains hydrogenase, cytochromes, DPNH-oxidase, phospholipid, and RNA (Cota-Robles, 1956; Alexander, 1956). Measuring the rate of appearance of these substances as submicroscopic particles should help to determine their origin. If these substances are released at the same rate as the disruption of the cell, it would suggest that the submicroscopic particles containing these substances exist as such in the cytoplasm. If the substances are released at a lower rate, the particles containing them cannot exist as such in the cytoplasm but must be formed by the disintegration of a larger structure. The rate of release of hydrogenase, one of the constituents of this particulate fraction, is shown in figure 4. Hydro-

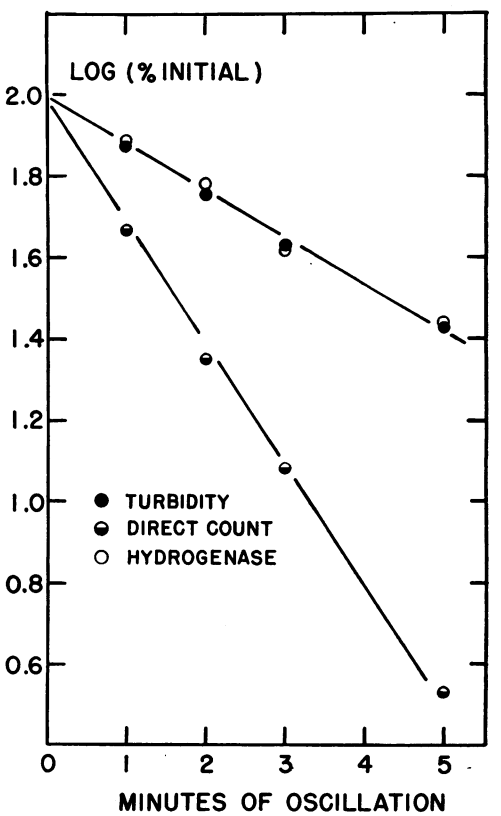

Figure 4. The rate of release of hydrogenase by sonic treatment. Hydrogenase as assayed in the supernatant liquid after centrifuging at 2,000 × G for 10 min and the amount sedimented was calculated by subtracting from the total hydrogenase. The logarithm of the per cent of the total hydrogenase still sedimentable has been plotted.

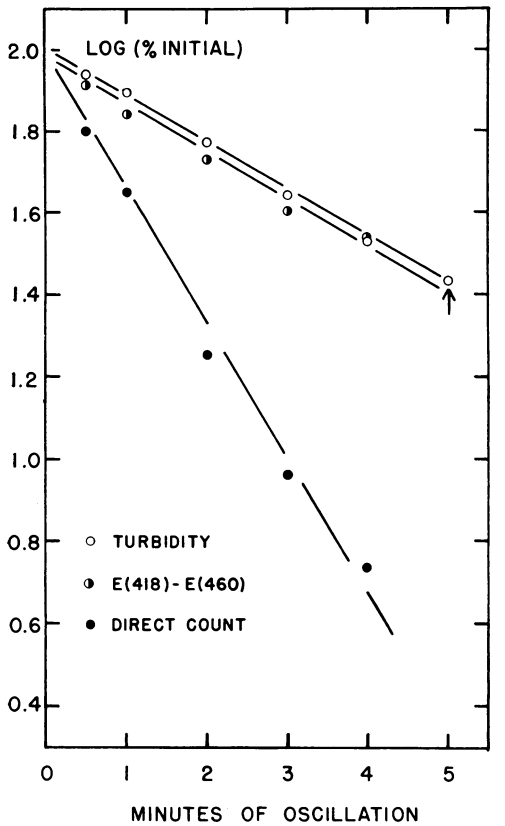

Figure 5. The rate of release of cytochrome by sonic treatment. Cytochrome was estimated in the supernatant liquid after centrifuging 10 min at 2,000 × G by measuring the difference in optical extinction between 418 and 460 mμ. The cytochrome still sedimentable was calculated by subtracting the amount in the supernatant liquid from a constant which adjusted the value at 5 min to the point indicated by the arrow.

genase was assayed in the supernatant after centrifuging at 2,000 × G for 10 min which removes all of the material of microscopic dimensions and essentially all of the material contributing to turbidity. The amount of the enzyme sedimented by this centrifugation was calculated by subtracting from the total hydrogenase which remains constant. Figure 4 shows that the rate of release of hydrogenase from the material sedimenting at 2,000 × G is identical with the rate of decrease in turbidity. This suggests that the submicroscopic particles containing hydrogenase are not present as such in the cytoplasm but are formed by the sonic disintegration of a larger structure.

Cytochrome, another constituent of the particulate fraction, is released at a rate which closely approximates the decrease in turbidity (figure 5). Cytochrome was measured in the supernatant after centrifuging at 2,000 × G; the cytochrome capable of sedimentation was estimated by subtracting the amount released from a constant such that the value at 5 min coincides with the turbidity.

Phospholipid, a third constituent of the particulate fraction, is released at the same rate as the decrease in turbidity (figure 6). Phospholipid was determined in the residue after centrifuging at 2,000 × G for 10 min. Since the particles containing hydrogenase, cytochrome and phospholipid are all released at the same rate as the decrease in turbidity, it is concluded that all three are in the same submicroscopic particle which is derived from the structure responsible for turbidity.

RNA, in contrast to the other constituents of the particulate fraction, is released at a rate approximately that of the decrease in direct count (figure 7). Since RNA is released more rapidly than the hydrogenase, cytochrome, and phospholipid, it can not be a part of the structure containing these substances. This nonidentity has been

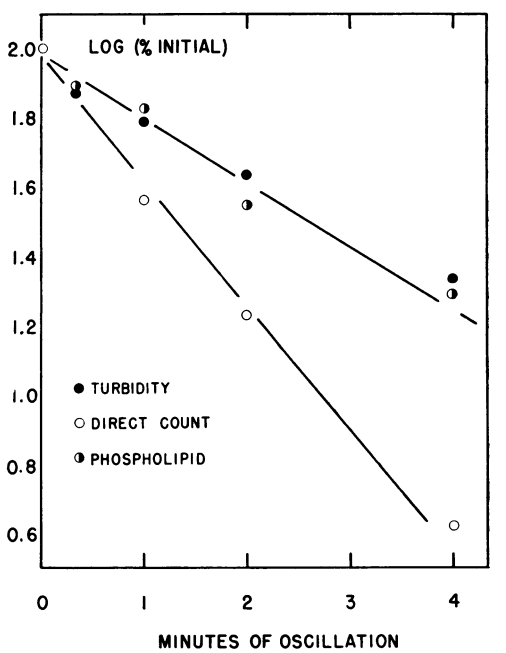

Figure 6. The rate of release of phospholipid by sonic treatment. Phospholipid was determined in the residue after centrifuging at 2,000 × G for 10 min. The logarithm of the per cent of the initial phospholipid has been plotted.

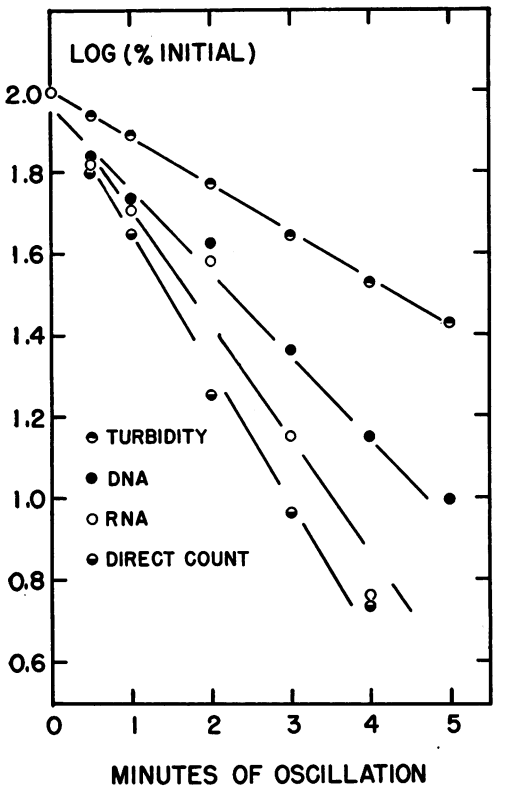

Figure 7. The rate of release of nucleic acids by sonic treatment. The nucleic acids were determined in the residue after centrifuging at 2,000 × G for 10 min. The logarithm of the per cent of the initial amount has been plotted.

verified by electrophoretic separation of the submicroscopic particles into two components; one component contains RNA and the other contains hydrogenase and cytochrome.

The rate of release of DNA is also shown in figure 7. This substance is released at a rate which appears to be exponential but does not correspond with either direct count or turbidity. A number of interpretations of this anomaly are possible; however, none has been tested. Approximately 75 per cent of the DNA of *A. vinelandii* is in the soluble fraction of cell free extracts (Cota-Robles, 1956; Alexander and Wilson, 1955).

DISCUSSION

The death of microorganisms exposed to intense sound has previously been found to be exponential (Hamre, 1949; Horwood *et al.*, 1950; Kinsloe *et al.*, 1954; Horton, 1953). From the observation that temperature has little effect on the rate of killing, Kinsloe *et al.* (1954) concluded that mechanical disruption was the mechanism of killing. The measurement of both total and viable cells during the sonic treatment of *A. vinelandii* demonstrates that cells are killed chiefly by mechanical disruption.

The primary action of sound on a cell of *A. vinelandii* is the disintegration of 40 per cent of the cell envelope into submicroscopic particles of varying sizes. This kills the cell and empties the residual cell envelope of its cytoplasm leaving a hull. These events are depicted in figure 8. The remaining hull is in turn disintegrated to submicroscopic particles by additional hits. The hull probably consists of a rigid wall and an adhering membrane.

The decrease in turbidity of a suspension of *A. vinelandii* exposed to sound results from the disintegration of cell envelopes to submicroscopic particles and is not due to the disruption of the cell. The ratio of the rate constant for change of

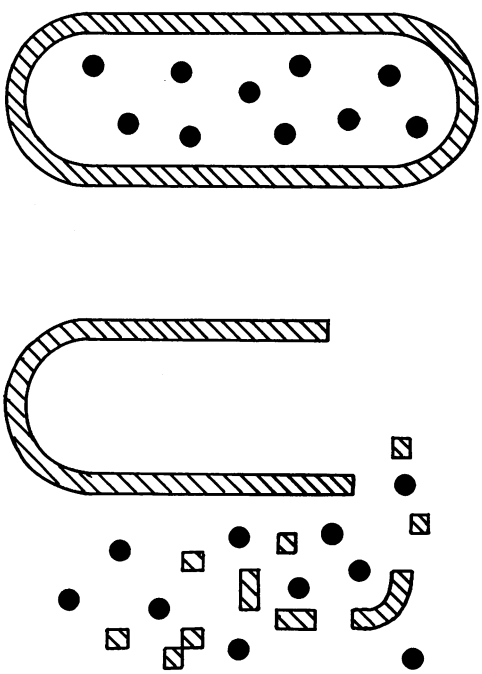

Figure 8. Schematic representation of a hit on a cell in the sound field. The circles represent constituents of the cytoplasm such as glucose-6-phosphate dehydrogenase and RNA. The cell's whole complement of these substances is released by the hit. The envelope is indicated by diagonal lines; approximately 40 per cent is disintegrated to submicroscopic particles by the hit.

turbidity to the rate constant for change in count (k_y/k_c) is the average fraction of the envelope destroyed by the first hit. Since the change in turbidity is exponential, the rate of comminution of the envelope to submicroscopic particles is independent of whether that envelope is intact (cell) or has suffered a previous hit (hull).

The submicroscopic particles obtained by ultracentrifugation of cell free extracts of bacteria frequently have been considered as homogeneous with respect to the composition of the individual particles and more frequently have been considered to have the same cytological origin (Bradfield, 1956). The particulate fraction of *A. vinelandii* is definitely heterogeneous; the component which contains the RNA is released at a rate which corresponds with the rate of disruption of the cells. A second component, of varying size but with constant chemical composition containing cytochrome, hydrogenase and phospholipid, is released at the same rate as the decrease in turbidity. These two types of particles can be distinguished in electron micrographs and have been separated by starch electrophoresis. Since all of the RNA is released when the cell is disrupted, it is likely that the submicroscopic particles which contain the RNA are present as such in the cytoplasm, and are not artifacts of disruption. Osmotic shock releases the particles which contain RNA but not those containing hydrogenase or cytochrome. The submicroscopic particles which contain hydrogenase, cytochrome, and phospholipid are derived from a larger structure. The coincidence in the rate of release of these substances with the rate of decrease in turbidity and the identification of hulls as the chief source of turbidity in excess of that resulting from remaining intact cells suggest that the particles containing hydrogenase, cytochrome and phospholipid are derived from the cell envelope. The purification of hulls formed by sonic treatment results in approximately 5-fold concentration of hydrogenase and phospholipid, and the purified hulls can be disintegrated by sound to submicroscopic particles. These findings identify the cell envelope as the parent substance from which the second type of submicroscopic particle is derived.

The application of this technique of differential release to the study of the structure of the bacterial cell can be extended to other microorganisms and to other methods of disruption. Slade and Vetter (1956) have studied the release of constituents of *Streptococcus pyogenes* by sonic treatment; their data have been plotted in figure 9. The rate of release of rhamnose is exponential but is only 23 per cent of the rate of decrease in viable count. The rate of decrease in sedimentable dry weight is a complex curve; it approaches the rate of decrease in viable count at the beginning and approaches the rate of release of rhamnose after 1 hr. This complex curve is given by a constituent common to both envelope and cytoplasm. The rates of release of the C and M antigens of *S. pyogenes* are considerably lower than the rate of killing and roughly parallel the release of rhamnose which suggests that the antigens are located in the cell envelope.

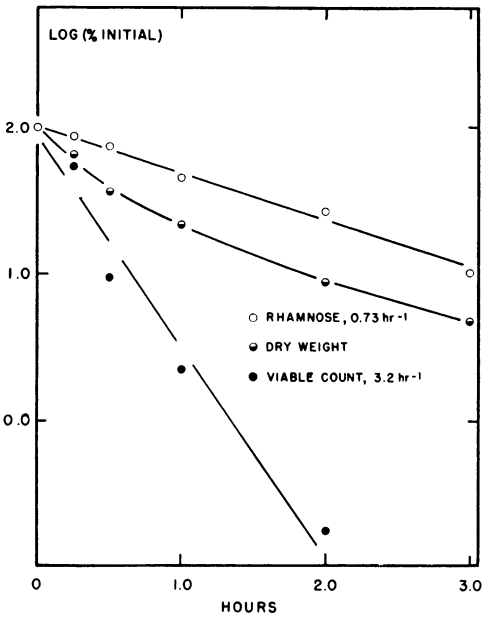

Figure 9. Kinetics of sonic disruption of *Streptococcus pyogenes*. The data of Slade and Vetter (1956) have been plotted logarithmically. The rhamnose released prior to extraction with bicarbonate was taken as the total.

The technique of differential release by sonic oscillation and by osmotic shock is being used in this laboratory in the study of the structure of bacteria, yeast and endospores. It should be a useful adjunct to the current methods of investigating bacterial cytochemistry.

SUMMARY

During the sonic treatment of suspensions of *Azotobacter vinelandii* the direct count and turbidity decrease exponentially. The total and

viable counts decrease at the same rate which indicates that the chief mechanism of killing is disruption of the cell. Turbidity decreases only 40 per cent as rapidly as the decrease in total count.

The turbidity in excess of that contributed by residual intact cells results from the scattering of light by large fragments of the broken cells. These fragments, which have been designated as "hulls," are empty but retain the shape and rigidity of the cell. Turbidity appears to be a function of the cell envelope and is independent of the rupture of the cell.

A "hit" on an intact cell disintegrates approximately 40 per cent of the surface to submicroscopic particles and empties the residual hull of cytoplasmic contents. The rates of release of glucose-6-phosphate dehydrogenase and RNA are identical with the rate of rupture of the cells which indicates that these substances are present in the cytoplasm. The rate of release of hydrogenase, cytochrome and phospholipid coincides with the decrease in turbidity which suggests that these substances are present in the cell envelope.

REFERENCES

ALEXANDER, M. 1956 Localization of enzymes in the microbial cell. Bacteriol. Revs., **20,** 67–93.

ALEXANDER, M. AND WILSON, P. W. 1955 Enzyme localization in *Azotobacter vinelandii*. Proc. Natl. Acad. Sci., U. S., **41,** 843–848.

ALLEN, R. J. L. 1940 The estimation of phosphorus. Biochem. J., (London), **9,** 858–865.

BRADFIELD, J. R. G. 1956 Organization of bacterial cytoplasm. In *Bacterial anatomy*, pp. 296–317. Edited by E. T. C. Spooner and B. A. D. Stocker. University Press, Cambridge, England.

COTA-ROBLES, E. H. 1956 The enzymatic organization of *Azotobacter vinelandii*. Ph.D. Thesis, University of California, Davis, California.

DISCHE, Z. 1930 Über einige neue charakteristische Farbreaktionen der Thymusnukleinsäure und eine Mikromethode zur Bestimmung derselben in Tierischen Organen mit Hilfe dieser Reaktionen. Mikrochemie, **8,** 4–32.

GEST, H. 1952 Properties of cell free hydrogenases of *Escherichia coli* and *Rhodospirillum rubrum*. J. Bacteriol., **63,** 111–121.

HAMRE, D. 1949 The effect of ultrasonic waves upon *Klebsiella pneumoniae*, *Saccharomyces cerevisiae*, *Miyagawanella felis* and influenza virus A. J. Bacteriol., **57,** 279–295.

HORTON, J. P. 1953 The effect of intermolecular bond strength on the onset of cavitation. J. Acoust. Soc. Amer., **25,** 480–484.

HORWOOD, M. P., HORTON, J. P., AND MINCH, V. A. 1950 Some effects of ultrasonic vibrations on *Escherichia coli*. Bacteriol. Proc., **1950,** p. 63.

KINSLOE, H., ACKERMAN, E., AND REID, J. J. 1954 Exposure of microorganisms to measured sound fields. J. Bacteriol., **68,** 373–380.

LINDSTRÖM, O. 1955 Physico-chemical aspects of chemically active ultrasonic cavitation in aqueous solutions. J. Acoust. Soc. Amer., **27,** 654–671.

MORTENSON, L. E. AND WILSON, P. W. 1954 Initial stages in the breakdown of carbohydrates by *Azotobacter vinelandii*. Arch. Biochem. and Biophys., **53,** 425–435.

MUDD, S. AND LACKMAN, D. B. 1941 Bacterial morphology as shown by the electron microscope. I. Structural differentiation within the streptococcal cell. J. Bacteriol., **41,** 415–420.

SCHACHMAN, H. K., PARDEE, A. B., AND STANIER, R. Y. 1952 Studies on the macromolecular organization of the microbial cell. Arch. Biochem. and Biophys., **38,** 245–260.

SCHNEIDER, W. C. 1945 Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and pentose nucleic acids. J. Biol. Chem., **161,** 293–303.

SLADE, H. D. AND VETTER, J. K. 1956 Studies on *Streptococcus pyogenes*. I. Observations on the microscopical and biological aspects of the disintegration and solubilization of a type 6 strain by sonic oscillation. J. Bacteriol., **71,** 236–243.