LOCATION OF ENZYMES IN *AZOTOBACTER AGILIS*

STANLEY A. ROBRISH AND ALLEN G. MARR

Department of Bacteriology, University of California, Davis, California

Received for publication July 28, 1961

ABSTRACT

ROBRISH, STANLEY A. (University of California, Davis) AND ALLEN G. MARR. Location of enzymes in *Azotobacter agilis*. J. Bacteriol. **83:** 158–168. 1962.—If the cells of *Azotobacter agilis* are disrupted by osmotic shock, respiratory enzymes and the compounds characteristic of cell wall and cytoplasmic membrane are recovered almost completely in large particles. The large particles obtained by osmotic shock were found by electron microscopy to consist of cell wall, cell membrane, and an internal membrane appearing as either vesicles or tubules in section. These envelopes are free of all the soluble cytoplasmic material and are essentially free of ribosomes.

Small particles obtained by osmotic shock are ribosomes; small particles obtained by sonic oscillation consist of both ribosomes and amorphous material, presumably fragments of the envelope.

Most methods of mechanical disruption of bacteria result in the recovery of respiratory enzymes in the centrifugal fraction containing submicroscopic particles (reviewed by Alexander, 1956; Mitchell, 1959; and Marr, 1960). Stanier (1954) predicted that the cell membrane was the locus of respiratory enzymes in bacteria. He based his prediction on the discovery by Weibull (1953) that ghosts obtained by osmotic lysis of protoplasts of *Bacillus megaterium* were rich in cytochromes.

Marr and Cota-Robles (1957) found that the submicroscopic particles containing respiratory enzymes were formed by comminution of a larger structure, presumably some part of the envelope of *Azotobacter agilis*. This paper will describe the disruption of *A. agilis* by direct osmotic shock, a technique which permits recovery of the cell envelope almost intact but emptied of cytoplasm, and will show that the respiratory enzymes are in this structure.

MATERIALS AND METHODS

Growth of cultures. A. agilis (*A. vinelandii* strain 0) was grown at 30 C in 10-liter bottles with 8 liters of Burk's medium modified by reducing the calcium concentration to prevent the formation of a precipitate (per liter: KH_2PO_4, 0.2 g; K_2HPO_4, 0.8 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.025 g; $FeNH_4(SO_4)_2 \cdot 12H_2O$, 0.0086 g; $Na_2MoO_4 \cdot 2H_2O$, 0.00025 g; and sucrose, 20 g. The culture was aerated through a porous stainless steel sparger (Micro Metallic Corporation, Glen Cove, N. Y.). The cells were harvested by centrifugation shortly after the end of exponential growth (30 hr), and washed free of slime by four successive centrifugations from distilled water at 2,000 $\times$ *g* for 45 min. After low speed centrifugation a loose overlayer of slime containing some cells was decanted from a pellet of well-packed cells and was discarded. Cells were either used immediately or stored at 4 C.

Cells labeled with P^{32} were obtained by growth in Burk's medium with one-fourth the normal amount of phosphate, which contained 10^7 count/min of P^{32}-orthophosphate. The cells were harvested by centrifugation and were washed by successive centrifugations from water. An appropriate quantity of cells labeled with P^{32} was added to a larger amount of nonradioactive cells for the estimation of phospholipid.

Chemical analyses. Hexosamine was determined colorimetrically by the method of Boas (1953) in samples hydrolyzed in 4 N HCl at 100 C for 15 hr. Chromatography of the hydrolyzate on Dowex-50 was unnecessary, since there were no interfering materials. The quantity of hexosamine was computed with reference to standards of glucosamine.

Rhamnose was determined by reaction with cysteine in sulfuric acid (Dische and Shettles, 1948). Samples were hydrolyzed in 1 N HCl at 100 C for 1 hr. After the HCl was removed by vacuum distillation, the hydrolyzate was washed

through a column of Dowex-50 (H^+ form) resin to remove interfering cations. After reaction the absorbancy was measured against a reagent blank at both 396 and 427 mμ. The quantity of rhamnose was computed from the difference in absorbancy at the two wavelengths. Since this color reaction is general for methyl pentoses, rhamnose was identified by descending paper chromatography of hydrolyzates on Whatman no. 1 paper in butanol-ethanol-water (52:32:16). The R_F of the methyl pentose in the sample corresponded to the R_F of an authentic sample of rhamnose.

Phospholipid was determined by measuring the radioactivity of ethanolic extracts, using cells labeled with P^{32}. The samples were precipitated with 5% trichloracetic acid (w/v), and extracted three times with ethanol at 60 C. A portion of the extract was evaporated on a planchet and counted. A sample was analyzed for total phosphorus (Allen, 1940) to determine the count/min per mg phosphorus; 1 mg of phosphorus in the ethanolic extract was taken to be the equivalent of 33 mg of phospholipid.

Ribonucleic acid (RNA) was determined by alkaline hydrolysis and adsorption of the resulting nucleotides on an anion exchanger. After elution the nucleotides were determined with orcinol. Analyses for RNA by orcinol reaction after fractionation by Schneider's (1945) procedure were inconsistent with measurements of ultraviolet absorbancy and acid-insoluble organic phosphorus. We, therefore, adapted the method of Cohn (1957) for quantitative, ion-exchange chromatography of ribonucleotides to isolate the total nucleotide fraction. A sample containing 0.05 to 0.5 mg RNA was digested in 0.3 N NaOH overnight at 37 C. The hydrolyzate was neutralized with Dowex-50 (H^+ form), and the resin was filtered and washed. The filtrate was titrated to pH 8 with tris(hydroxymethyl)-aminomethane (tris). The sample was transferred to a 1.8 by 2 cm column of Dowex-1 (200 to 400 mesh) resin which had been previously equilibrated with 0.01 M tris-Cl buffer at pH 8. After the application of the sample, the column was washed with 10 ml of 0.01 M tris-Cl buffer, pH 8.6, and the washings were discarded. The nucleotides were eluted with 10 ml of 1 N HCl, and were estimated by the orcinol reaction (Mejbaum, 1939). The difficulties we encountered in the estimation of RNA from bacterial extracts were also found by de Deken-Grenson and de Deken (1959), who developed a similar method.

Enzymatic assays. Hydrogenase was determined manometrically, using methylene blue as an oxidant (Marr and Cota-Robles, 1957). Samples were treated and maintained under an atmosphere of H_2 at all stages prior to assay, to prevent the inactivation of hydrogenase (Green and Wilson, 1953). One unit of enzyme was taken to be that amount required to oxidize 1 μmole of H_2 per min at 30 C.

Cytochrome was measured by the difference in absorbancy at 414 mμ (Soret maximum of the principal cytochrome) and 390 mμ. The samples were treated 5 min at 4 C under an atmosphere of H_2 at full output in a Raytheon sonic oscillator to decrease light scattering. Samples were reduced with sodium borohydride, and the absorbancy was measured against a water blank. The mount of cytochrome is expressed as the difference in absorbancy per cm at 414 and 390 mμ.

Reduced diphosphopyridine nucleotide (DPNH) oxidase was assayed by the decrease in absorbancy at 340 mμ resulting from the oxidation of DPNH. A 1-cm absorption cell contained 2.9 ml of 0.05 M tris-Cl buffer, pH 8.6, and the enzyme (50 μliters or less), which was added with a microsyringe. The reaction was started by the addition of 0.1 ml of a solution containing 2 mg DPNH per ml. One unit of enzymatic activity is defined as that amount of enzyme required to change the absorbancy 1.0 per min.

Glucose 6-phosphate dehydrogenase was determined by the rate of increase of absorbancy at 340 mμ resulting from the reduction of diphosphopyridine nucleotide (DPN) by glucose 6-phosphate. A 1-cm absorption cell contained 0.2 ml of 0.02 M glucose 6-phosphate, 0.1 ml of the enzyme, and 2.5 ml of 0.0033 M $MgSO_4$ in 0.05 M tris-Cl, pH 8.6. The reaction was started by the addition of 0.2 ml of a solution containing 10 mg DPN per ml. One unit of enzymatic activity is defined as the amount of enzyme required to change the absorbancy 1.0 per min.

Adenosine deaminase was measured as the rate of decrease in absorbancy at 265 mμ as adenosine is converted to inosine. A 1-cm absorption cell contained 0.6 ml of enzyme, 2.2 ml of 0.05 M tris-Cl (pH 8.6), and 0.2 ml of 0.0006 M adenosine. The reaction was started

by the addition of the adenosine. One unit of enzymatic activity is defined as the amount of enzyme required to change the absorbancy 1.0 per min.

All of the spectrophotometric measurements of reaction rate were made in a Beckman DU spectrophotometer adapted for recording linearly in absorbancy at fixed wavelength (Marr and Marcus, *in press*). The temperature was maintained at 30 C by water from a constant temperature bath flowing through a heat exchanger in the cell compartment.

Electron microscopy. Pseudoreplicas were prepared essentially according to the method of Hall (1956). The sample was sprayed on freshly cleaved mica, shadowed with Pt at an angle of 18°, and overlayered with a film of carbon. The carbon film was floated on water and picked up on copper grids for observation. Samples to be observed air-dried were dispersed with a Brinkman 1 Mc emulsifier (Brinkman Instruments, Great Neck, N. Y.), deposited on a Formvar grid from a mist obtained with a Brinkman 3 Mc nebulizer, and shadowed with uranium at an angle of 11°. Thin sections were made of samples fixed in osmium tetroxide; the fixed samples were embedded in polymethylmethacrylate and sectioned with a Porter Blum ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.) fitted with a glass knife (Chapman, 1959). All samples were photographed with an RCA EMU 3 electron microscope.

Osmotic shock. A centrifuged pellet of cells was mixed with an equal volume of 3 M glycerol. After 5 min the mixture was drawn into a syringe and slowly ejected into 10 volumes of mechanically stirred 0.05 M tris-Cl buffer (pH 7.5), containing 0.001 M $MgSO_4$, at 4 C (Robrish and Marr, 1957). This buffer was chosen to stabilize ribosomes (Gillchriest and Bock, 1958; Bolton, Hoyer, and Ritter, 1958). Less than 4% of the cells remained intact. After disruption, the preparation was treated with 0.5 μg crystalline deoxyribonuclease per ml and allowed to stand at room temperature for 20 min. This treatment reduces the viscosity, which would otherwise interfere with subsequent centrifugal separation.

Sonic disruption of cells. A 40-ml suspension of cells in 0.05 M tris-Cl (pH 7.5) containing 0.001 M $MgSO_4$ was treated at full power output for 5 min in a 10 kc Raytheon magnetostrictive sonic oscillator at 0 to 3 C under an atmosphere of H_2. Less than 0.5% of the cells remained intact.

Centrifugal separation. The disrupted cells were separated arbitrarily into large particles, small particles, and soluble fraction by the following method of differential centrifugation. The extract was centrifuged at 10,000 $\times$ *g* for 15 min; the resulting pellet was washed once by recentrifugation from the same buffer used for disruption. This pellet is termed "large particles." The supernatant liquid and washings were combined and centrifuged at 100,000 $\times$ *g* for 2 hr; the resulting pellet was recentrifuged from buffer. The second pellet is termed "small particles." The supernatant liquid and washings from this step are termed "soluble fraction."

RESULTS

Location of enzymes. If the small particles containing respiratory enzymes found after sonic disruption of cells arise from the envelope (Marr and Cota-Robles, 1957), the disruption of cells by osmotic shock, which opens but does not comminute the envelope, should allow a quantitative recovery of the respiratory enzymes in the low-speed centrifugal fraction which contains the emptied envelopes.

Equal samples of washed cells were disrupted by osmotic shock and by sonic treatment. The materials from the disrupted cells were separated by centrifugation into large particles, small particles, and soluble fraction, and were assayed for various enzymic activities (Table 1). The hydrogenase, DPNH oxidase, and cytochrome are recovered mainly in the small particles after sonic treatment but in the large particles after osmotic shock. Two soluble enzymes, glucose 6-phosphate dehydrogenase and adenosine deaminase, were released equally by the two methods of disruption. Thus, the recovery of the respiratory enzymes in the large particles after osmotic shock is in accord with the assumption that these enzymes are contained in the envelope. The release of the soluble enzymes indicates that the disruption of the cells by osmotic shock is complete.

Two components of the cell walls of bacteria, rhamnose and hexosamine (Salton, 1956), were recovered almost completely in the large particles after osmotic shock, but were distributed between the large and small particles after sonic disruption (Table 2). Phospholipid, a likely constituent of both the membrane (Gilby, Few, and McQuillin, 1958) and the cell wall (Westphal

TABLE 1. *Distribution of enzyme activity*

Enzyme	Osmotic disruption				Sonic disruption			
	Total units*	Large particles†	Small particles	Soluble	Total units*	Large particles	Small particles	Soluble
		%	%	%		%	%	%
Hydrogenase	3.75	69	3	0	3.12	30	60	0
DPNH oxidase	0.743	82	12	0	1.05	5	100	0
Cytochrome	0.00565	93	0	0	0.00725	9	64	15
Glucose 6-phosphate dehydrogenase	0.239	0	0	100	0.239	0	0	100
Adenosine deaminase	0.159	0	0	100	0.159	0	0	100

* The total is the activity of the unfractionated extract in units per mg dry wt. The units are defined in Materials and Methods.

† Large particles sediment at 10,000 × *g* for 15 min, and small particles at 100,000 × *g* for 2 hr; the soluble material is that which does not sediment at 100,000 × *g* for 2 hr.

TABLE 2. *Distribution of chemical components*

	Osmotic disruption				Sonic disruption			
	Total*	Large particles	Small particles	Soluble	Total*	Large particles	Small particles	Soluble
		%	%	%		%	%	%
Phospholipid	120	103	4	3	119	35	62	16
Rhamnose	18.1	82	8	3	17.4	31	52	14
Hexosamine	11.0	82	7	18	10.8	49	46	16
RNA	72.5	15	56	28	65.7	7	66	27

* The total of each constituent (mg per g dry wt) is based on analysis of unfractionated extracts and is taken as 100%.

et al., 1958), was also recovered in the large particles after osmotic shock, and distributed between the large and small particles after sonic disruption. A much larger fraction of the rhamnose, hexosamine, and phospholipid than of the cytochrome and DPNH oxidase are found in the large particles after sonic disruption.

The distribution of RNA in the centrifugal fractions is essentially independent of the method of disruption (Table 2). Approximately 60% of the RNA appears in the small particles and 30% in the soluble fraction. Osmotic shock, compared with sonic disruption, results in slightly more RNA in the large particles and correspondingly less RNA in the small particles.

Morphology of the large particles. Since the compounds characteristic of bacterial cell walls were recovered in the large particles after osmotic shock, this fraction would be expected to contain the cell wall and any other structures of the cell which do not escape. Figure 1a is an electron micrograph of the large particles obtained by osmotic shock after air-drying and shadowing. The fraction consists of structures, approximately the same size and shape as the original cell, which appear to be envelopes enclosing material dense to the electron beam. Some regions are quite transparent to electrons, but other regions are more opaque than purified cell walls (Salton and Horne, 1951).

Figure 1b is an electron micrograph of a thin section of large particles obtained by osmotic shock. The outer layer of the structure is presumed to be the cell wall, and the inner layer (M), which in some regions is well separated from the wall, is presumed to be cytoplasmic membrane. The wall is obviously torn in sections of some of the envelopes (O). All the envelopes show an internal structure which appears most commonly as empty vesicles (V) of varying diameter. In less damaged envelopes the internal structure is tubular (T). This internal structure is restricted to the area circumscribed by the membrane; it is absent in the region between the wall and membrane if these two structures have separated. We assume that the regions of high opacity

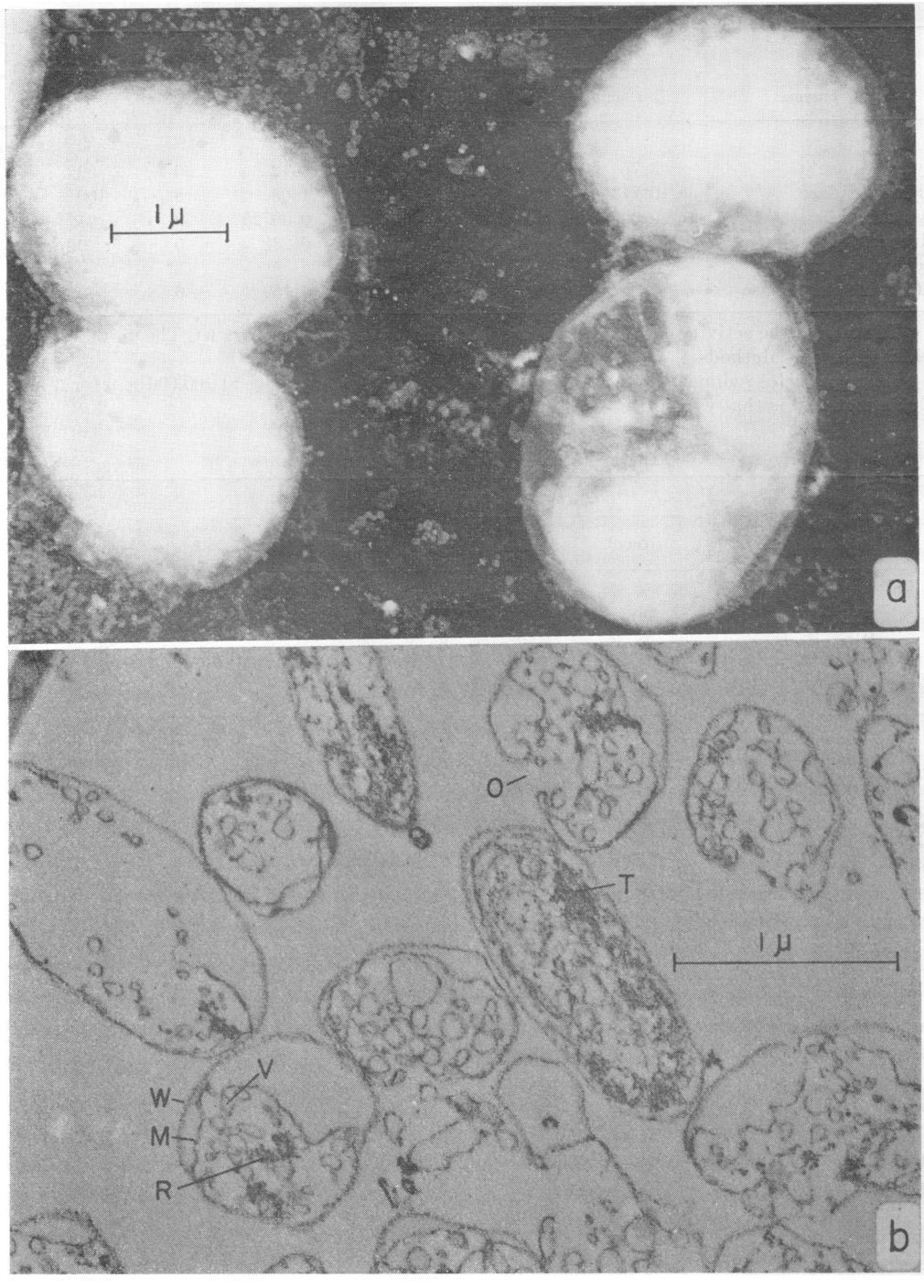

FIG. 1. *Large particles after osmotic shock. (a) Pseudoreplica of an air-dried specimen. (b) Thin section. The cell wall (W) appears torn in some of the sections (O). The outer cell membrane (M) is partially detached from the wall. Internal membrane is evident within areas bounded by the outer cell membrane but absent between the wall and membrane. The internal membrane most commonly appears as vesicles (V); however, in less damaged envelopes it appears as tubules (T). A few envelopes contain dark granules which are probably ribosomes (R).*

to electrons of the air-dried envelope result from the membrane and the internal structure that it encloses and that the more transparent regions result from the retraction of the membrane from the wall.

Figure 2 is an electron micrograph of the large particles obtained by sonic disruption. The particles are flat and transparent to the electron beam; the particles are of various sizes, all of which are much smaller than the envelopes obtained by osmotic shock.

Small particles. Electron micrographs of the small particles obtained by the two methods of disruption are shown in Fig. 3. The small particles obtained by osmotic shock (Fig. 3a) are spherical to polygonal. The larger particles appear to be aggregates of one particle of intermediate size and two smaller particles. The major axes of the three sizes of particles measured from the electron micrograph are 42, 56, and 70 mμ, respectively. The small shadows suggest considerable flattening. The size and appearance of the small particles obtained by osmotic shock are those of ribosomes (Hall and Slayter, 1959).

The small particles obtained by sonic disruption (Fig. 3b) include a considerable amount of amorphous material in addition to the ribosomes.

Electrophoresis. Since the electron micrographs showed amorphous material as well as ribosomes in the small particles obtained by sonic treatment, the particles obtained by the two methods of disruption were examined by analytical electrophoresis. The small particles obtained by osmotic shock were resolved into three components (Fig. 4a) all of which move anodically. Most of the material is in the leading peak, which has a mobility of -8.75 cm^2 per sec^{-1} per v^{-1} at pH 7.5; this mobility is approximately that expected for ribosomes (Petermann, Hamilton, and Mizen, 1954). The particles obtained by sonic treatment (Fig. 4b) have the same leading component; however, they show additional major components of low mobility. The schlieren pattern in Fig. 4b is obscured by the light scattering of the components which remain near the origin. Visual observation revealed at least two peaks in the region obscured in the photograph by light scattering; the combined area of these

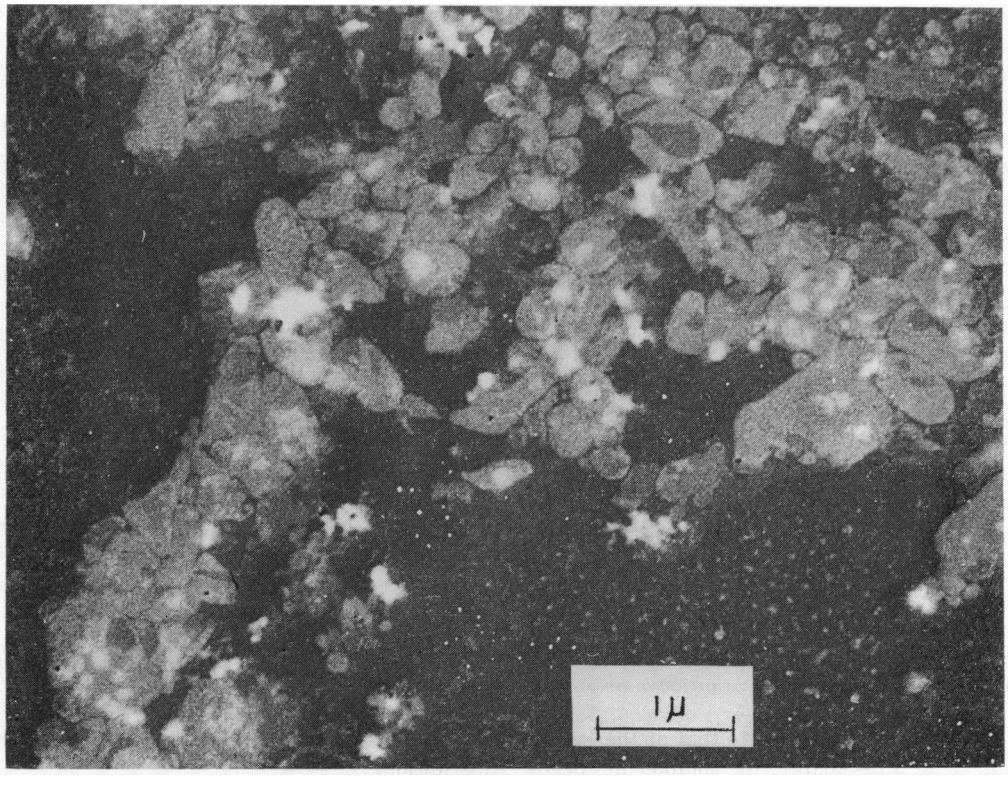

FIG. 2. *Large particles after sonic treatment. The photograph is a pseudoreplica of an air-dried specimen.*

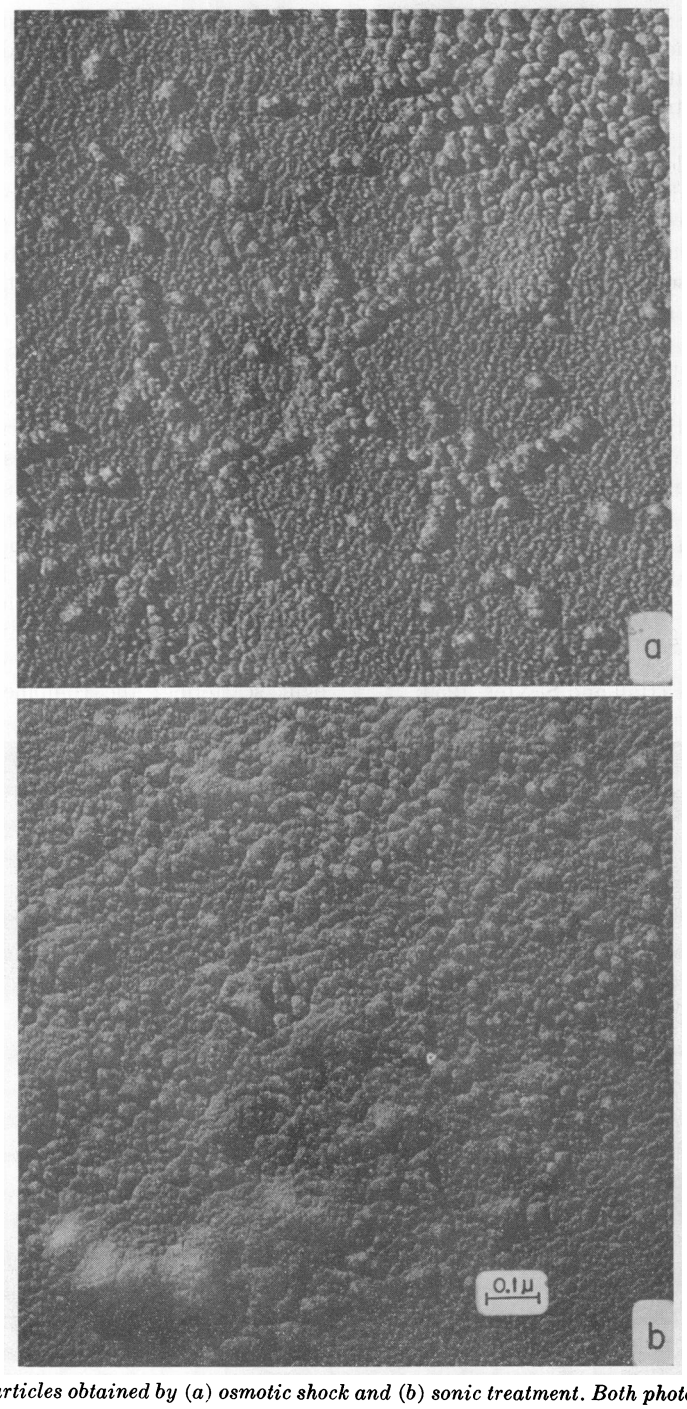

FIG. 3. *Small particles obtained by (a) osmotic shock and (b) sonic treatment. Both photographs are of air-dried samples shadowed with uranium at an angle of 11°.*

two peaks was comparable to that of the leading component. Presumably, the components of low mobility correspond with the amorphous material which distinguished the electron micrographs of the small particles prepared by sonic disruption.

Ultracentrifugation. Figure 5a shows an ultracentrifugal analysis of the small particles pre-

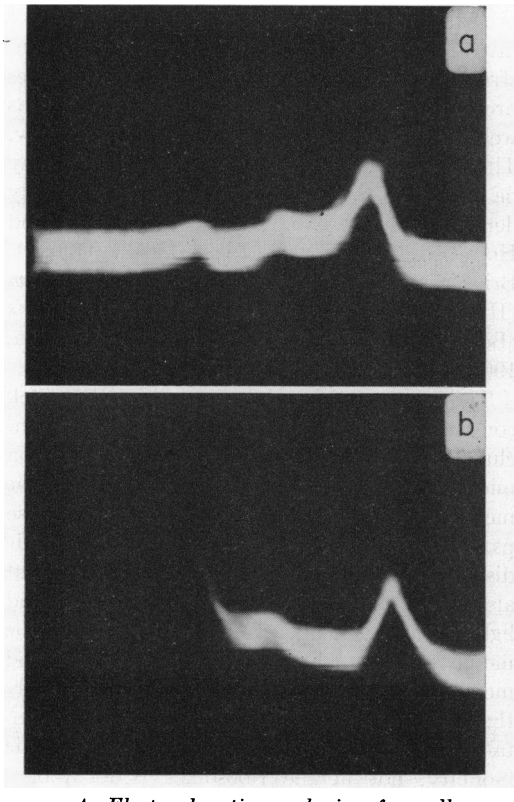

FIG. 4. *Electrophoretic analysis of small particles. The concentration of particles was approximately 5 mg per ml in 0.05* M *tris-Cl (pH 7.5) containing 0.001* M $MgSO_4$ ($\Gamma/2 = 0.043$). *The electrophoretic cell had a cross-sectional area of 3 cm² and was operated at a current of 5 ma. Both photographs are of ascending boundaries taken with a diagonal slit and cylindrical lens (magnification 1.9 ×). The initial boundary was formed at the extreme left of the pattern. Migration toward the anode is indicated by movement from left to right. (a) Particles prepared by osmotic shock photographed after 34 min. The leading component has a mobility of 8.75 × 10^{-5} cm^2 sec^{-1} $volts^{-1}$. (b) Particles prepared by sonic treatment photographed after 49 min. The leading component has a mobility of 7.37 × 10^{-5} cm^2 sec^{-1} $volts^{-1}$. The left portion of the pattern is obscured by components which scatter light. Two peaks were visually evident in this region.*

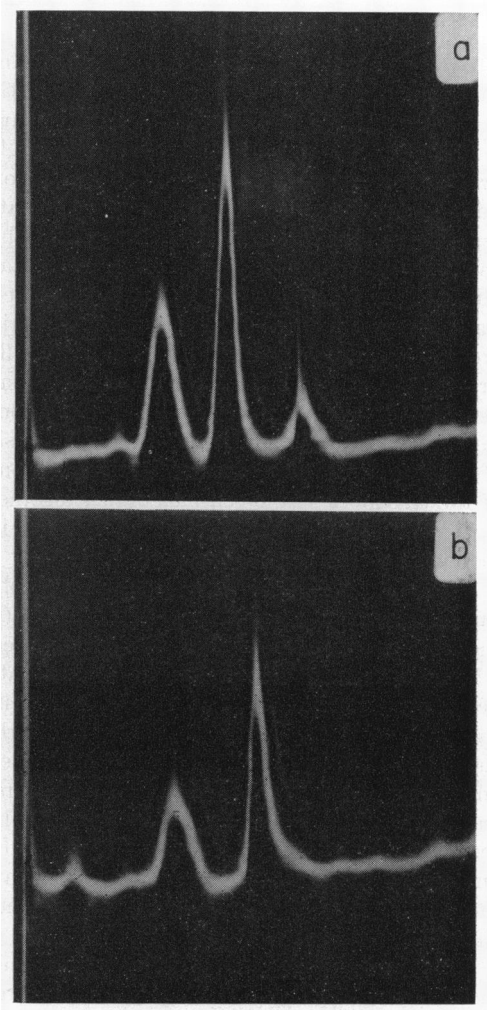

FIG. 5. *Analysis of the small particles by ultracentrifugation. Particles at a concentration of approximately 5 mg per ml in the same buffer used for electrophoresis (Fig. 4) were centrifuged at 10 C in a Spinco Model E analytical ultracentrifuge. The photographs were made with a diagonal slit and cylindrical lens 16 min after the rotor reached a maximal speed of 42,000 (magnification 5.1 ×). (a) Particles obtained by osmotic shock; the major peaks from left to right have sedimentation constants of 31S, 49S, and 67S, respectively, uncorrected for concentration. (b) Particles obtained by sonic treatment; the two major peaks have sedimentation constants of 33S and 51S, uncorrected for concentration.*

pared by osmotic shock. Three major components are evident, with sedimentation constants of 67S, 49S, and 31S (uncorrected for concentration). These values correspond with the three size classes of ribosomes obtained from *A. agilis* by Gillchriest and Bock (1958). Only two major components are evident in the particles prepared by sonic treatment, with sedimentation constants of 51S and 33S. No boundary was observed corresponding to material present in the particles prepared by sonic treatment but absent in particles prepared by osmotic shock.

DISCUSSION

Hydrogenase, DPNH oxidase, and cytochromes, as well as the compounds characteristic of the cell wall, rhamnose and hexosamine, are recovered in the large particles if the cells of *A. agilis* are disrupted by osmotic shock. In electron micrographs, the large particles appear as flattened envelopes; however, the opacity of the envelope to electrons is much greater than that observed by others for purified cell walls. Cell walls prepared from gram-negative bacteria are more opaque to electrons than cell walls prepared from gram-positive bacteria; this has been attributed to undefined cytoplasmic contamination (Salton and Horne, 1951). The basis of the high opacity is revealed by thin sections of the envelopes as the cell membrane, together with an internal membrane which appears in section as vesicles or tubules.

The loss of cytoplasm from the envelopes obtained by osmotic shock is evident from (i) the complete release of two soluble enzymes, adenosine deaminase and glucose 6-phosphate dehydrogenase, and of the soluble RNA, (ii) the lack of cytoplasmic matrix, particularly the lack of ribosomes, in most of the sectioned envelopes, and (iii) the almost complete release of particulate RNA by osmotic shock. The respiratory enzymes, which are retained in the envelope, could not be located in small, unattached particles dispersed like the ribosomes throughout the cytoplasm.

Since the envelopes emptied of cytoplasmic matrix by osmotic shock are complex structures composed of at least three distinguishable components, wall, cell membrane, and an internal structure, we must face the question of which of these houses the respiratory enzymes. After sonic disruption, the components of the cell wall are found in both large and small particles, yet the DPNH oxidase and cytochrome are absent in the large particles. The resolution of wall components from these enzymes suggests that the cell wall is not the locus of the enzymes; however, a more direct demonstration is desirable.

Either the outer cell membrane or the internal membrane or both could be the locus of the respiratory enzymes. The internal membrane could be a structure passively trapped in the envelopes opened by osmotic shock, distortions of the cell membrane induced by the osmotic disruption, or extensions of the cell membrane pre-existing before osmotic disruption. A further investigation of this problem, which demonstrates the internal membrane in envelopes prepared by several methods of disruption as well as in intact cells, will be published separately. The internal membrane in the azotobacter may be related to the internal membranes previously demonstrated in streptomycetes (Glauert and Hopwood, 1959; Stuart, 1959), bacilli (Glauert, Brieger, and Allen, 1961), *Rhodospirillum rubrum* (Hickman and Frenkel, 1959), *Alcaligenes faecalis* (Beer, 1960), and blue-green algae (Shatkin, 1960; Hopwood and Glauert, 1960).

The small particles obtained by osmotic shock consist almost exclusively of ribosomes, a conclusion based both on the appearance of electron micrographs and on the high electrophoretic mobility of the major component of these particles. The small particles obtained by sonic disruption contain not only the ribosomes but also particles of various sizes and shapes and light-scattering components of low electrophoretic mobility, presumably fragments of the wall and membrane. It has been demonstrated previously that hydrogenase and cytochrome are contained in the components of low electrophoretic mobility, not in the ribosomes (Cota-Robles, Marr, and Nilson, 1958; Tissières et al., 1959). The small particles obtained from bacteria are analyzed most commonly by ultracentrifugation. Because of the appearance in the ultracentrifuge of a predominant boundary concommitant with a particular enzymatic activity in the pellet from centrifugation, it has frequently been assumed that the enzymatic activity is located in the sedimenting species which forms the boundary. Several enzymatic activities were identified with a 40S component on this basis by Schachman, Pardee, and Stanier (1952). Similar interpretations for enzymes and chemical constituents have been made subsequently (Billen and Volkin, 1954; Saperstein and Starr, 1955; Hunt, Rodgers, and Hughes, 1959). It is evident from Fig. 5 that the nonribosomal components in the small particles obtained by sonic treatment do not result in additional boundaries in the ultracentrifuge. The fragments of wall and membranes would be expected to have a wide range of sizes and shapes, which precludes the formation of boundaries. Thus, ultracentrifugation would be unreliable in detecting the presence of such components.

In more recent experiments, an early intermediate in protein synthesis (McQuillen, Roberts, and Britten, 1959) and small amounts of enzymes (Aronson et al., 1960) are reported to be bound to ribosomes. The hazard of random fragments of wall and membrane contaminating the preparation, even after centrifugation in a density gradient, should be eliminated by electrophoretic separation. One cannot rely on ultracentrifugal analysis as a criterion of purity of ribosomes.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid 4261 and 9860 from the National Science Foundation.

We acknowledge the extensive help given us by Jack Pangborn of the Electron Microscope Laboratory.

LITERATURE CITED

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

ALLEN, R. J. L. 1940. The estimation of phosphorus. Biochem. J. **34**:858–865.

ARONSON, A. I., E. T. BOLTON, R. J. BRITTEN, D. B. COWIE, J. D. DUERKSEN, B. J. MCCARTHY, K. MCQUILLEN, AND R. B. ROBERTS. 1960. Carnegie Inst. Wash. Year Book, p. 229–289.

BEER, M. 1960. Disposition of membranes in *Alcaligenes faecalis*. J. Bacteriol. **80**:659–664.

BILLEN, D., AND E. VOLKIN. 1954. The effect of X rays on the macromolecular organization of *Escherichia coli*. J. Bacteriol. **67**:191–197.

BOAS, N. F. 1953. Method for the determination of hexosamines in tissues. J. Biol. Chem. **204**:553–563.

BOLTON, E. T., B. H. HOYER, AND D. B. RITTER. 1958. Stability of ribonucleoprotein particles of *Escherichia coli*, p. 18–21. *In* R. B. Roberts, [ed.], Microsomal particles and protein synthesis. Pergamon Press, New York.

CHAPMAN, G. B. 1959. Electron microscopy of ultrathin sections of bacteria. III. Cell wall, cytoplasmic membrane and nuclear material. J. Bacteriol. **78**:96–104.

COHN, W. E. 1957. Methods of isolation and characterization of mono and polynucleotides by ion exchange chromatography, p. 724–743. *In* S. P. Colowick and N. O. Kaplan, [ed.], Methods in enzymology. Academic Press Inc., New York.

COTA-ROBLES, E. H., A. G. MARR, AND E. H. NILSON. 1958. Submicroscopic particles in extracts of *Azotobacter agilis*. J. Bacteriol. **75**:243–252.

DE DEKEN-GRENSON, M., AND R. H. DE DEKEN. 1959. Elimination of substances interfering with nucleic acids estimation. Biochim. et Biophys. Acta **31**:195–207.

DISCHE, Z., AND L. B. SHETTLES. 1948. A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem. **175**:595–603.

GILBY, A. R., A. V. FEW, AND K. MCQUILLEN. 1958. The chemical composition of the protoplast membrane of *Micrococcus lysodeikticus*. Biochim. et Biophys. Acta **29**:21–29.

GILLCHRIEST, W. C., AND R. M. BOCK. 1958. Isolation and characterization of bacterial nucleoprotein particles, p. 1–10. *In* R. B. Roberts, [ed.], Microsomal particles and protein synthesis. Pergamon Press, New York.

GLAUERT, A., E. M. BRIEGER, AND J. M. ALLEN. 1961. The fine-structure of vegetative cells of *Bacillus subtilis*. Exptl. Cell Research **22**:73–85.

GLAUERT, A., AND D. A. HOPWOOD. 1959. A membranous component in the cytoplasm in *Streptomyces coelicolor*. J. Biophys. Biochem. Cytol. **6**:515–516.

GREEN, M., AND P. W. WILSON. 1953. Hydrogenase and nitrogenase in *Azotobacter*. J. Bacteriol. **65**:511–517.

HALL, C. E. 1956. Visualization of individual macromolecules with the electron microscope. Proc. Natl. Acad. Sci. U. S. **42**:801–806.

HALL, C. E., AND H. S. SLAYTER. 1959. Electron microscopy of ribonucleoprotein particles from *E. coli*. J. Molecular Biol. **1**:329–332.

HICKMAN, D. D., AND A. W. FRENKEL. 1959. The structure of *Rhodospirillum rubrum*. J. Biophys. Biochem. Cytol. **6**:277–284.

HOPWOOD, D. A., AND A. M. GLAUERT. 1960. The fine-structure of the nuclear material of a blue-green alga, *Anabaena cylindrica* Lemm. J. Biophys. Biochem. Cytol. **8**:813–823.

HUNT, A. L., A. RODGERS, AND D. E. HUGHES. 1959. Sub-cellular particles and the nicotinic acid hydroxylase system in extracts of *Pseudomonas flourescens* KB1. Biochim. et Biophys. Acta **34**:354–372.

MCQUILLEN, K., R. B. ROBERTS, AND R. J. BRITTEN. 1959. Synthesis of nascent protein by ribosomes in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. **45**:1437–1447.

MARR, A. G. 1960. Enzyme localization in bacteria. Ann. Rev. Microbiol. **14**:241–260.

MARR, A. G., AND E. H. COTA-ROBLES. 1957. Sonic disruption of *Azotobacter vinelandii*. J. Bacteriol. **74**:79–86.

MEJBAUM, W. 1939. Über die bestimmung kleiner pentosemengen, insbesondere in derivaten

der adenylsaure. Z. physiol. Chem., Hoppe-Seyler's **258**:117–120.

MITCHELL, P. 1959. Biochemical cytology of microorganisms. Ann. Rev. Microbiol. **13**:407–440.

PETERMANN, M. L., M. G. HAMILTON, AND N. A. MIZEN. 1954. Electrophoretic analysis of the macromolecular nucleoprotein particles of mammalian cytoplasm. Cancer Research **14**:360–366.

ROBRISH, S. A., AND A. G. MARR. 1957. Osmotic disruption of azotobacter. Bacteriol. Proc., p. 130.

SALTON, M. R. J. 1956. Bacterial cell walls, p. 81–110. *In* E. T. C. Spooner and B. A. D. Stocker, [ed.], Bacterial anatomy. University Press, Cambridge.

SALTON, M. R. J., AND R. W. HORNE. 1951. Studies of the bacterial cell wall. II. Methods of preparation and some properties of cell walls. Biochim. et Biophys. Acta **7**:177–197.

SAPERSTEIN, S., AND M. P. STARR. 1955. Association of carotenoid pigments with protein components in non-photosynthetic bacteria. Biochim. et Biophys. Acta **16**:482–488.

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

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

SHATKIN, A. J. 1960. A chlorophyll-containing cell fraction from the blue-green alga, *Anbaena variabilis*. J. Biophys. Biochem. Cytol. **7**:583–584.

STANIER, R. Y. 1954. Some singular features of bacteria as dynamic systems, p. 3–24. *In* E. Racker, [ed.], Cellular metabolism and infections. Academic Press, Inc., New York.

STUART, D. C. 1956. Fine-structure of the nucleoid and internal membrane systems of streptomyces. J. Bacteriol. **78**:272–281.

TISSIÈRES, A., J. D. WATSON, D. SCHLESSINGER, AND R. R. HOLLINGWORTH. 1959. Ribonucleoprotein particles from *Escherichia coli*. J. Molecular Biol. **1**:221–233.

WEIBULL, C. 1953. Characterization of the protoplasmic constituents of *Bacillus megaterium*. J. Bacteriol. **66**:696–702.

WESTPHAL, O., O. LÜDERITZ, E. EICHENBERGER, AND E. NETER. 1958. Mucopolysaccharides of Gram-negative bacteria: newer chemical and biological aspects, p. 187–199. *In* G. E. W. Wolstenholme and M. O'Connor, [ed.], Chemistry and biology of mucopolysaccharides. J. and A. Churchill, Ltd., London.