SUBMICROSCOPIC PARTICLES IN EXTRACTS OF *AZOTOBACTER AGILIS*[1]

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Attempts to describe the location of enzymes in a bacterial cell have been generally unsuccessful because the methods used have been indirect. Alexander (1956) has recently reviewed the literature pertaining to this subject. The discovery that certain enzymes in bacterial extracts are easily sedimented by centrifugation has resulted in these enzymes being correctly designated as particulate. Most investigators have inferred that submicroscopic particles found in extracts exist as such in the cytoplasm of the cell. It is equally possible that these submicroscopic particles may be derived from a larger structure.

The work described in this report has shown that a particulate fraction of *Azotobacter agilis* (*Azotobacter vinelandii*) contains two types of particles which differ in chemical composition, enzymatic function, physical structure, and origin. One type contains hydrogenase, cytochrome, and phospholipid and is produced by fragmentation of the cell envelope; a second type is a ribonucleoprotein which exists as discrete particles in the cytoplasm.

METHODS

Cultures. Seven L cultures of *Azotobacter agilis* (*Azotobacter vinelandii* strain O) were grown in Burk's medium for 24 to 30 hr at 30 C. The cells were recovered by centrifugation and washed 3 times by centrifuging from distilled water at 2000 × G for 10 min. The packed cells were stored at −10 C. The details of the methods of cultivation are given by Marr and Cota-Robles (1957).

Sonic disruption. Forty ml of a suspension of cells in 0.05 M phosphate buffer, pH 6.8, containing 200 mg wet weight of cells per ml was treated in a 10 kc Raytheon sonic oscillator at 1.20 to 1.33 amp, audio frequency (50 to 70 acoustical watts). The gas phase was H_2 and the temperature was maintained below 3 C during treatment. Unless stated otherwise, the suspension was treated for 5 min, which is sufficient to reduce the turbidity to less than 25 per cent of the initial value.

Chemical determinations. Acid-soluble phosphorus, phospholipid, and nucleic acids were determined by the method of Schneider (1945). Acid-soluble phosphorus was extracted with cold trichloracetic acid; phospholipid was extracted with 95 per cent ethanol and was estimated from the phosphorus in the extract.

Nucleic acid phosphorus was extracted with hot trichloracetic acid. Total nucleic acid was determined both by extinction of the extract at 260 mμ and from the phosphorus content of the extract. Deoxyribonucleic acid (DNA) was determined by reaction with diphenylamine (Dische, 1930) or with *p*-nitrophenylhydrazine (Webb and Levy, 1955).

Phosphorus was determined after digestion with perchloric acid by the method of Allen (1940).

Ethanolamine was identified and determined as the N-2,4-dinitrophenyl derivative formed by reaction with 2,4-dinitrofluorobenzene (Hayaishi and Kornberg, 1954).

Nitrogen was determined by a slight modification of the method of Hillier *et al.* (1948); after digestion of the sample the ammonia was distilled into 0.1 N H_2SO_4, and the distillate nesslerized.

Carbohydrate was measured with anthrone using glucose as a standard (Morris, 1948).

Protein was determined by the Biuret reaction (Weichselbaum, 1946) or by reaction with the Folin-Ciocalteu reagent (Lowry *et al.*, 1951).

Glucose was determined by oxidation with ferricyanide. A 1.0 ml sample containing 0.2 to 0.8 μmole of glucose was mixed with 1.0 ml of 7×10^{-3} M $K_3Fe(CN)_6$ and 1.0 ml of 0.15 M Na_2CO_3 containing 2.3×10^{-3} M KCN. This mixture was heated in boiling water for 10 min and the residual

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ferricyanide was measured by determining the extinction at 420 mμ. Five moles of ferricyanide are reduced per mole of glucose.

Zone electrophoresis was performed on beds of starch according to the method of Paigen (1956). *Swan* potato starch was washed repeatedly by suspension in distilled water until the effluent was free of solutes absorbing in the ultraviolet. A slurry of the starch made in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.1 was added to lucite troughs 33 by 4 by 2 cm to give a layer 1 cm deep. The starch bed was connected by filter paper wicks to compartments containing a stainless steel cathode and platinum anode. After the starch had drained sufficiently to solidify, a 1 cm band was cut from the starch. The cut was filled with a solution of the sample in 0.001 M Tris to which sufficient starch was added to make a thick slurry. The lower ionic strength in the segment containing the sample results in a pronounced sharpening of the front as the colloids move into adjacent zones of higher ionic strength. The electrode compartments were filled with buffer and a potential of 300 v was applied for 16 hr. During the separation, the starch beds were cooled to 5 to 10 C by a flow of water around the lucite trough. After the separation the starch bed was cut into 1 cm segments and the materials eluted from the starch with water or buffer.

Enzymatic assays. Hydrogenase was assayed by the manometric measurement of the reduction of methylene blue by hydrogen at 30 C. Each vessel contained 0.5 ml of 0.016 M methylene blue, 0.5 to 1.0 ml of enzyme in buffer and 0.05 M phosphate buffer at pH 6.8 to make 3.0 ml.

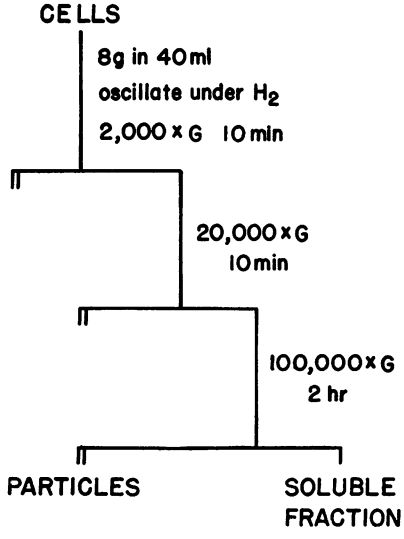

Figure 1. Fractionation of extracts of *Azotobacter agilis* by differential centrifugation.

The oxyhydrogen reaction was measured by omitting methylene blue and substituting a gas phase of 0.9 atm H_2 and 0.1 atm air.

Oxidases of reduced diphosphopyridine nucleotide (DPNH) and reduced triphosphopyridine nucleotide (TPNH) were assayed by measuring the oxygen consumed when rate-limiting concentrations of the oxidase were coupled with a suitable dehydrogenase. Glucose-6-phosphate dehydrogenase. Glucose-6-phosphate dehydrogenase prepared from *A. agilis* (Mortenson and Wilson, 1954) was used to generate either DPNH or TPNH. In some experiments alcohol dehydrogenase prepared from yeast was used to generate DPNH.

Glucose-6-phosphate dehydrogenase was assayed by measuring the rate of reduction of DPN spectrophotometrically (Mortenson and Wilson, 1954). The cuvettes contained 0.1 ml of enzyme, 0.5 ml of freshly prepared 1 per cent $NaHCO_3$, 10 mg DPN and water to 3.0 ml. The extinction at 340 mμ was measured with a Beckman spectrophotometer. It was necessary to inactivate the DPNH oxidase with 10^{-3} M KCN or to remove the interfering oxidase by centrifugation for 2 hr at 100,000 × G which does not remove a measurable quantity of the dehydrogenase.

Catalase was assayed by measuring manometrically the evolution of O_2 from H_2O_2 (Clark, 1952).

Hexokinase as measured by determining the disappearance of glucose from a system which contained the following: 2.5 ml of 0.02 M glucose, 0.5 ml of 0.1 M $MgCl_2$, 1.0 ml of 0.04 M adenosine triphosphate (ATP), 1.0 ml of 0.1 M Tris at pH 8.1 and 1.0 ml of enzyme. Residual glucose was measured after glucose-6-phosphate and protein were precipitated with equimolar $Ba(OH)_2$ and $ZnSO_4$.

The oxidation of intermediates of the tricarboxylic acid cycle was measured under essentially the same conditions as those used by Stone and Wilson (1952).

Adenosine deaminase was assayed by measuring the change in extinction at 265 mμ. Cuvettes contained 0.2 ml of 6×10^{-4} M adenosine, 2.7 ml 0.1 M Tris at pH 8.3 and 0.1 ml of

enzyme. The enzyme was diluted to give a rate of 0.01 units of extinction per min.

Esterification of phosphorus during the oxidation of hydrogen was measured by trapping the ATP with hexokinase and glucose. The amount of ATP formed was measured by determining the residual glucose as in the assay for hexokinase.

RESULTS

The differential centrifugation of sonic extracts of *Azotobacter agilis* is described in figure 1. Those enzymes found in the residue after centrifuging for 2 hr at 100,000 × G are designated as particulate and are quite easily distinguished from the soluble enzymes which are not measurably sedimented after this treatment. Table 1 summarizes the distribution of enzymes between the crude particles and soluble fraction.

Enzymatic activity of the soluble fraction. The final supernatant liquid has a large number of enzymatic activities. Hexokinase, glucose-6-phosphate dehydrogenase, catalase and adenosine deaminase were found exclusively in this fraction. Alexander and Wilson (1956) have demonstrated fumarase, aconitase, isocitric dehydrogenase, and α-ketoglutaric dehydrogenase in this fraction.

Enzymatic activities of the crude particles. Most of the hydrogenase of the azotobacter can be recovered in the crude particles. Table 2 shows that 81 per cent of the hydrogenase of the cell extract is recovered in the particles and that the specific activity is increased twofold over that of the original extract.

The red-brown color of the particles results from a high content of cytochrome. The particles show pronounced absorption bands at 418, 520–530, and 550–565 mμ which correspond to the bands of reduced cytochromes *b* and *c*. These bands disappear on aeration and reappear if the preparation is bubbled with hydrogen or supplied with DPNH. The particles were found to contain the complete system for the oxidation of DPNH and TPNH by oxygen.

In addition to hydrogenase, the cytochromes and both of the pyridine nucleotide oxidases, the particles contain the enzymes which oxidize succinate and malate but do not oxidize other intermediates of the tricarboxylic acid cycle. Alexander and Wilson (1956) have demonstrated cytochrome oxidase in the particles obtained from this organism.

The crude particles also esterify phosphate during the oxidation of hydrogen (table 3) which confirms the report by Hyndman *et al.* (1953) of

TABLE 1

Enzymatic composition of the particles and soluble fraction

Particles	Soluble Fraction
DPNH and TPNH oxidases	Hexokinase
Cytochromes	Adenosine deaminase
Malic and succinic oxidases	Glucose-6-P dehydrogenase
Oxidative phosphorylation	Aconitase*
	Isocitric dehydrogenase*
	Ketoglutaric dehydrogenase*

* Alexander and Wilson (1956).

TABLE 2

The recovery of hydrogenase after centrifugal separation of sonic extracts

Fraction	mg N per ml	μL H_2 per ml per hr	Specific Activity	Per Cent Recovery
Extract	6.72	16,100	2,400	100
Crude particles	2.47	13,100	5,300	81
Soluble fraction	4.02	2,600	640	16

TABLE 3

Phosphorylation during the oxidation of hydrogen by crude particles

	μA Oxygen	μmoles Glucose	P/O
Complete	6.3	0.714	0.11
Complete	5.3	1.11	0.21
− Particles	0	0.020	—
+ 5 mM ATP	—	4.85	—

* Oxygen computed as one-third of total gas consumed.

Warburg vessels contained the following final concentrations of reactants: glucose, 10^{-2} M; $MgCl_2$, 5×10^{-3} M; adenosine diphosphate, 10^{-2} M; inorganic phosphate, 1×10^{-2} M; tris, 1.7×10^{-2} M; hexokinase, 1 mg per ml, and sufficient crude particles to obtain a rate of approximately 5 μL gas ($H_2 + O_2$) per minute. The pH was 8.1, the gas phase was 0.9 atm H_2 and 0.1 atm air, and the temperature 30 C.

TABLE 4

Phosphorus compounds in the particles and soluble fraction

Fraction	Total P	Acid Soluble P	Lipid P	Nucleic Acid P
Extract	18.7	6.75	3.36	7.55
Particles	9.37	1.05	2.67	4.70
Soluble fraction	10.0	6.10	0.81	1.89

The data are mg of phosphorus recovered from 40 ml of extract which contained 129 mg N.

the phosphorylation of adenosine diphosphate during the oxidation of hydrogen by extracts of *A. agilis*. The P/O ratio varied from 0.1 to 0.36 with different preparations; the P/O ratio did not change materially after storing at −10 C for one week. The low P/O ratio can not be attributed to ATP-ase which was scarcely measurable in the crude particles; most of the ATP-ase was in the soluble fraction.

Chemical composition of the particles. The chemical analyses of the crude particles and of the soluble fraction are summarized in table 4. The particles contain about one-half of the total phosphorus and one-third of the Kjeldahl nitrogen of the cell extract. The particles contain most of the nucleic acid and phospholipid while the supernatant contains most of the acid soluble phosphorus of the cell extract.

Table 5 shows the composition of the crude particles and of the soluble fraction as per cent of dry weight. The two fractions were first dialyzed against distilled water and were then lyophilized. These results show that the nucleic acid of the particles is ribonucleic acid (RNA); deoxyribonucleic acid (DNA) is found chiefly in the soluble fraction. The particles contain 19.5 per cent phospholipid calculated from the phosphorus in the ethanol extract. Thus, two unique chemical properties of the crude particles are the high content of phospholipid and RNA.

TABLE 5

The chemical composition of the particles and soluble fraction after dialysis

Substance in Per Cent Dry Weight	Particles	Soluble Fraction
Phospholipid	19.5	0.15
Total nucleic acid	10.4	3.0
DNA	1.0	3.0
Total phosphorus	2.2	0.9
Total nitrogen	10.6	9.7

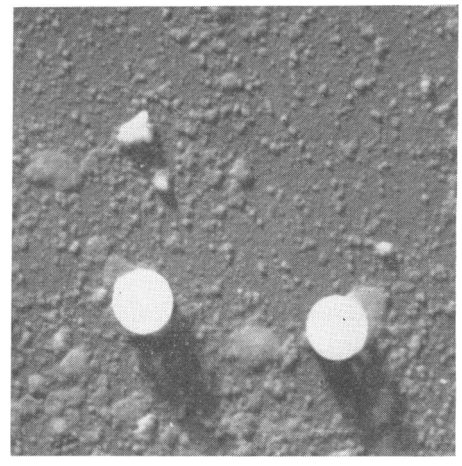

Figure 2. Electron micrograph of crude particles. The specimen was lyophilized and shadowed with uranium. The electron-dense spheres are polystyrene latex 260 mμ in diameter.

Fractionation of crude submicroscopic particles. A determination of the distribution of enzymes and other substances between the particulate and soluble fractions fails to answer two important questions:

(1) Do the particles exist as such in the cytoplasm or are they derived from some larger structure?

(2) Is the particulate fraction homogeneous or does it consist of chemically and enzymatically distinct particles?

These questions have been answered in part by following the rates of release of components of the crude particles during sonic disruption of cells (Marr and Cota-Robles, 1957). One constituent, RNA, was released at a rate identical with the rate of disruption of the cells, which suggests that the RNA is located in the cytoplasm. A second group of constituents, hydrogenase, cytochrome and phospholipid, was released at a lower rate than disruption, indicating that the particles containing hydrogenase, cytochrome and phospholipid are derived by the comminution of a larger cellular structure. The crude particles must contain at least two types of particles; one containing RNA and one or more containing hydrogenase, cytochrome, and phospholipid.

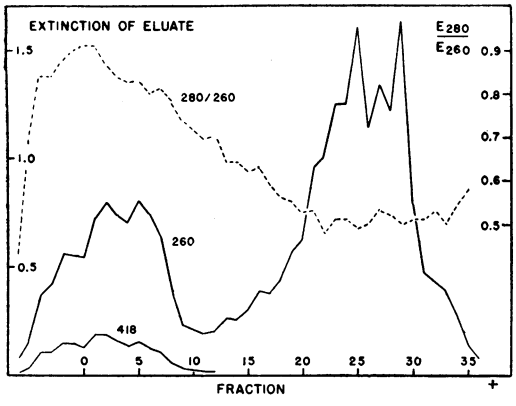

Figure 3. Electrophoretic separation of crude particles on starch. The abscissa is in cm from the origin toward the anode. The fractions from −8 to 10, which are referred to in the text as the slow moving component, contain cytochrome and hydrogenase. The fractions from 20 to 30, which are referred to as the fast moving component, contain ribonucleoprotein.

Electron micrographs of the crude particles reveal at least two types of particles (figure 2). One type is dense to electrons and has a uniform diameter of 30 mμ. A second type has a low density and varies in size. The electron micrographs as well as the kinetics of release of cellular components suggest that two types of particles are present in the particulate material of cell extracts.

The crude particles have been separated into two fractions by zone electrophoresis on starch. The ultraviolet absorption of the eluates of various zones of the starch is plotted in figure 3. The slower-moving component has a yellow-red color and absorbs at 418 mμ indicative of cytochromes. This component contains hydrogenase but contains essentially no RNA.

The rapidly moving component with a constant 280/260 ratio of 0.5 is well separated from the colored component. The ultraviolet absorption spectrum of this component is shown in figure 4; the maximum is at 258 mμ and the minimum is at 234 mμ. The rapidly moving component contains approximately 50 per cent RNA and 50 per cent protein based on dry weight. It contains no measurable DNA. Thus this component is a nucleoprotein. The homogeneity of the nucleoprotein was tested by free-boundary electrophoresis. Figure 5 shows that the nucleoprotein is heterogeneous; at least two peaks can be detected which show reversible spreading. Ultracentrifugal analysis also shows this material to be heterogeneous (figure 6). The major component may be identical with the 40 S particles described by Schachman *et al.* (1952), since it has a sedimentation constant of 42 S.

The nucleoprotein particles have none of the enzymatic activities that have been demonstrated in the crude particles. Some preparations were contaminated with ribonuclease which hydrolyzes the RNA into dialyzable nucleotides leaving an insoluble, basic protein.

Isolation of hulls. Microscopic examination revealed that during sonic oscillation a large structure transiently accumulates (Marr and Cota-Robles, 1957). This large structure, which we have called the hull, can be isolated and purified. Hulls contain the cell wall and may also contain other components which adhere to the wall.

Hulls have been prepared in sufficient quantity for enzymatic, chemical, and physical characterization. Cells washed free of slime are oscillated

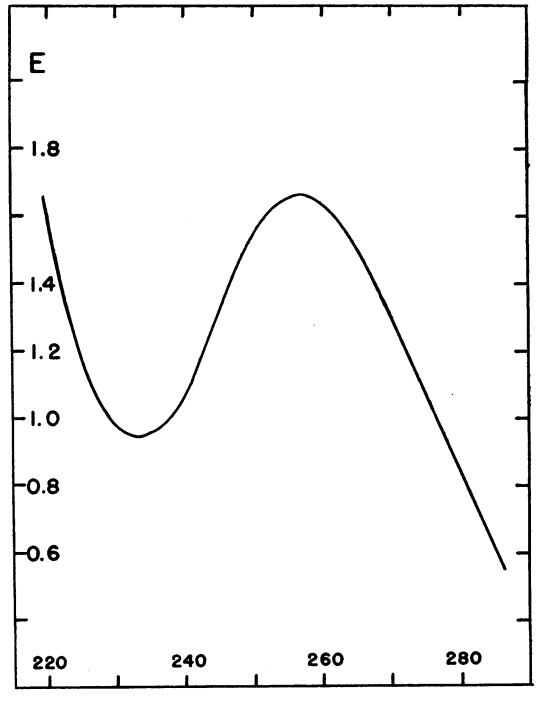

Figure 4. Ultraviolet absorption spectrum of the ribonucleoprotein prepared by electrophoretic separation of the crude particles.

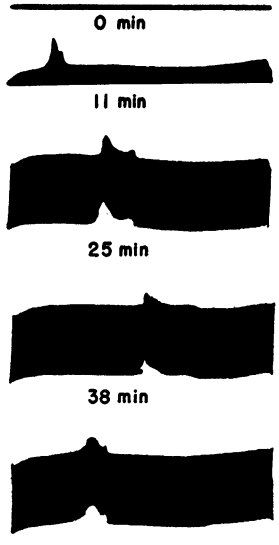

Figure 5. Schlieren patterns of the purified ribonucleoprotein during free-boundry electrophoresis; the microcell contained 0.01 M phosphate buffer, pH 6.8 and the current was 10 mA. The poor resolution of the peaks results in part from the light scattering by the preparation which limited the concentration of colloid to less than 1 per cent.

until the turbidity of the suspension has decreased only 50 per cent which has been found to give a maximum yield of hulls. Centrifugation at 2000 × G for 10 min sediments both unbroken cells and hulls; however, the cells pack firmly, and the hulls are layered over the cells in a loose mass which can be decanted. Iterated centrifugation removes essentially all of the unbroken cells. Centrifugation at 20,000 × G sediments the hulls into a firm pellet, which permits the hulls to be washed free of soluble constituents and submicroscopic particles.

Enzymatic activities of hulls. The purified hulls have all of the enzymatic activities previously described in crude particles. The specific activity of the hydrogenase in whole cells is 1800, while the specific activity of the hydrogenase of purified hulls is 11,000; thus, the specific activity is increased over 5-fold merely by separating the hulls from the rest of the contents of the cell. Oxyhydrogen activity, DPNH and TPNH oxidases, cytochromes, and malic and succinic oxidases are also contained in the hulls. The increase in specific activity suggests that these enzymes are integral components of the hulls.

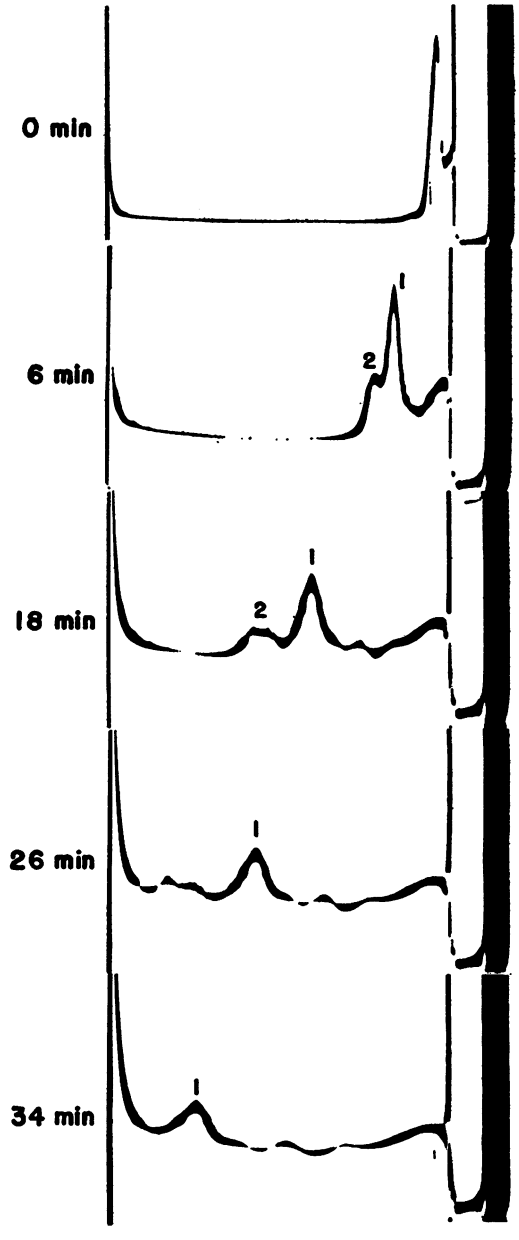

Figure 6. Schlieren patterns of the purified ribonucleoprotein during ultracentrifugation. The preparation had been dialyzed against 0.02 M NaCl. The largest peak has a sedimentation constant of 42 S.

None of the enzymatic activity is lost if the hulls are washed with water, buffer, or 1 M NaCl.

The purified hulls also esterify phosphate during the oxidation of hydrogen (table 7). The P/O ratio varied from 0.13 to 0.3. Since the hull

has suffered considerably less mechanical damage than the submicroscopic particles, it seems unlikely that this is the basis of the low P/O ratio of the crude particles.

The reduction of tetrazolium dyes has been used to indicate the "sites of oxidation-reduction activity" of bacterial cells (Mudd, 1953). Cells of *A. agilis* reduce neotetrazolium and deposit the colored formazan as granules in the cell. Figure 7 is a record of the appearance of a group of cells which were reducing neotetrazolium with glucose as the substrate. The photograph reveals that the granules of formazan gradually increase in size; in one cell the granules finally coalesce into a larger granule. Figure 8 shows the granules of formazan that appear during the reduction of neotetrazolium hulls with hydrogen as a substrate. If the focus is altered during microscopic examination the granules appear to be deposited on the inner surface of the hulls. No unattached granules of formazan were observed. Before the addition of neotetrazolium the hulls were free of visible granules. The deposition of formazan in both cells and hulls appears to be independent of pre-existing, microscopic granules.

Chemical composition of the hulls. The hulls contain protein, carbohydrate, and phospholipid but contain no measurable RNA (table 6). The hulls contain 3 per cent DNA which represents

TABLE 6

The chemical composition of hulls of Azotobacter agilis

Substance	Per Cent Dry Weight
Protein	60
Carbohydrate	16
Phospholipid	16-20
DNA	3
RNA	0

TABLE 7

Phosphorylation during the oxidation of hydrogen by hulls

μA Oxygen	μmoles Glucose	P/O
1.30	0.30	0.22
1.33	0.20	0.15
6.00	1.95	0.31

Conditions are the same as those in table 3 except hulls were substituted for crude particles.

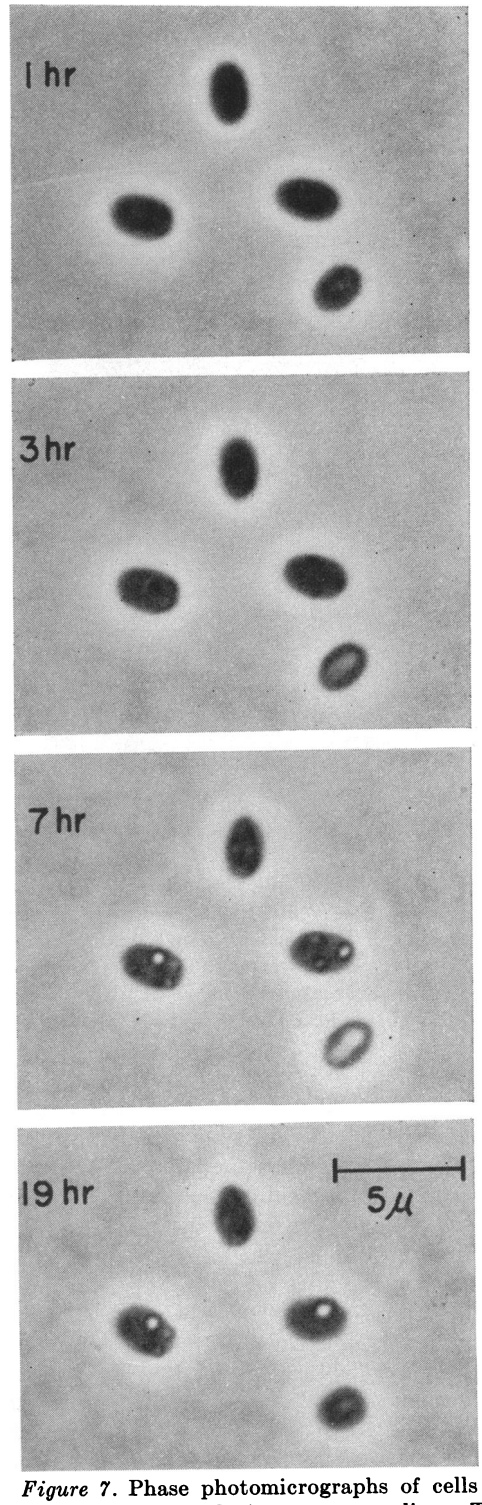

Figure 7. Phase photomicrographs of cells of *Azotobacter agilis* reducing neotetrazolium. The cells were suspended in agar containing 1 per cent glucose and 0.1 per cent neotetrazolium chloride.

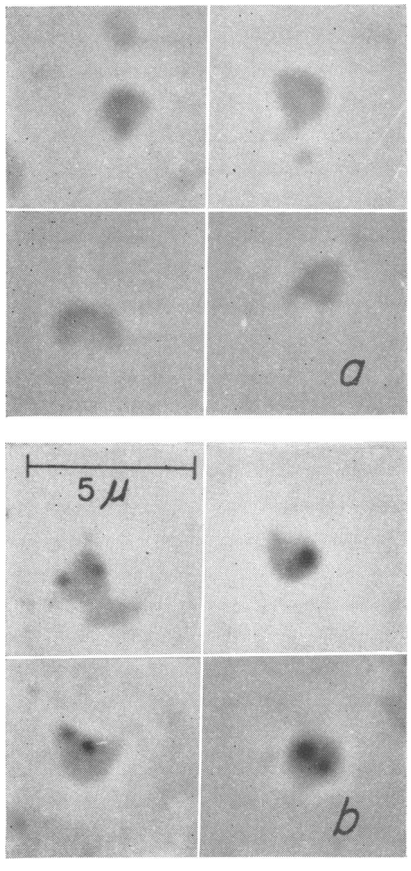

Figure 8. Phase photomicrographs of hulls before and after reduction of neotetrazolium. A suspension of hulls in phosphate buffer containing 0.1 per cent neotetrazolium was placed in a tube with a gas atmosphere of H_2; *a* shows the hulls initially and *b* shows the hulls after 60 min under H_2.

approximately 20 per cent of the DNA of the cells from which the hulls were prepared. We have not determined whether the DNA is adsorbed to a structural component of the hull or whether the hull occludes nuclear fragments.

The phospholipid which comprises 16 to 20 per cent of the hulls has been found to contain ethanolamine but no choline or serine. A typical analysis of the ethanol extract of hulls is as follows: Kjeldahl nitrogen, 1.36 microatoms; ethanolamine, 1.32 μmoles; phosphorus, 0.84 microatoms. All of the nitrogen is accounted for as the amino group of ethanolamine. Cholesterol could not be detected in hulls.

Based on the 5-fold increase in the specific activity of hydrogenase in the hull compared with the whole cell and a comparable increase in the concentration of phospholipid, one can estimate that the hull makes up about 20 per cent of the dry weight of a cell.

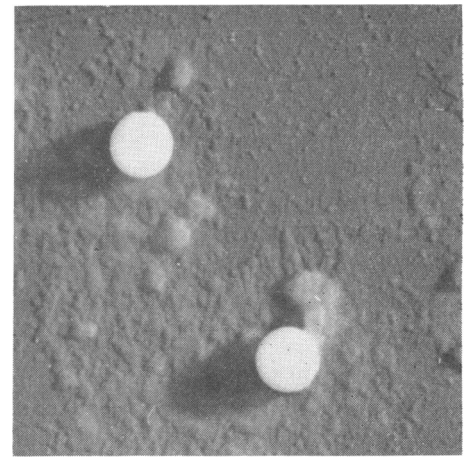

Figure 9. Electron micrograph of particles obtained by sonic treatment of purified hulls. The preparation of the specimen and subsequent photography was identical with that in figure 2.

Sonic fragmentation of hulls. Sonic treatment of purified hulls produces fragments which are irregular in size and shape and have low density to electrons (figure 9). These fragments appear identical to the fragments of low density in electron micrographs of crude particles (figure 3). Extending sonic oscillation for 1 hr produces fragments which do not sediment if centrifuged for 2 hr at 100,000 × G; however, individual proteins can not be resolved from the mixture. Extending sonic treatment seems to fragment the hulls into progressively smaller particles, each containing a full complement of enzymes.

DISCUSSION

The submicroscopic particles in sonic extracts of *A. agilis* consist of two distinct types which can be distinguished in electron micrographs and separated by electrophoresis. The particles which contain RNA are released by sonic treatment at the same rate as the disruption of the cell which suggests that these particles exist as such in the cytoplasm (Marr and Cota-Robles, 1957). Since the major component of the ribonucleoprotein has a sedimentation constant of 42 S, it is likely that the "40 S" particles found by Schachman *et al.* (1952) in a wide variety of bacteria are similarly particles of ribonucleoprotein. These

may be the particles observed by Bradfield (1956) in electron micrographs of thin sections. The metabolic significance of the particles of ribonucleoprotein is unknown; none of the enzymatic activities studied were found in these particles. Because of the circumstantial evidence for a function of RNA in the synthesis of protein (Brachet, 1955), we are investigating the role of these particles in the synthesis of protein by the azotobacter. A component of rat liver microsomes which, also, is half protein and half RNA has been implicated in protein synthesis (Littlefield *et al.*, 1955).

The hull comprises the cell wall, evidenced by its shape and rigidity, and in addition it contains other substances which can not be mechanically separated from the wall. It is possible that the hull is also the structure responsible for the selective permeability of the cell. At the moment there is no evidence for a physically separate wall and plasma membrane in the azotobacter. Fragments of cell wall can not be distinguished from fragments of plasma membrane as in micrococci (Mitchell and Moyle, 1956), nor can a retraction of the protoplast from a surrounding wall be demonstrated in plasmolysis as in *Bacillus megaterium* (Weibull, 1953*a*). The high content of phospholipid is characteristic of structures which control selective permeability (Parpart and Ballentine, 1952).

The hull has been shown to contain hydrogenase and cytochrome and to esterify phosphate during the oxidation of hydrogen. These findings suggest that the hull is the site of terminal respiration. Hulls free of visible granules reduce neotetrazolium with hydrogen and form granules of formazan which confirms the finding of Weibull (1953*b*) that the granules themselves are not the site of reduction.

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SUMMARY

Two types of submicroscopic particles in sonic extracts of *Azotobacter agilis* (*Azotobacter vinelandii* have been separated by zone electrophoresis. One type is a particle of ribonucleoprotein with a diameter of 30 mμ and a sedimentation constant of 42 S. A second particle appears to be a fragment of the hull. Purified hulls obtained by brief sonic oscillation contain hydrogenase, cytochrome, phospholipid and the requisite enzymes for oxidative phosphorylation.

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