A Method for Preparing Membrane Vesicles from *Acetobacter aceti*

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Membrane vesicles were prepared from *Acetobacter aceti* by a method entailing growth in the presence of glycine, osmotic shock and lysozyme digestion. Energy-dependent transport of the amino acid valine could be demonstrated in the vesicles.

INTRODUCTION

For studies on acetate transport in *Acetobacter aceti* membrane vesicles that are unable to metabolize acetate were required. This paper describes the preparation and the characterization of such vesicles.

METHODS

Organism and cultivation. *Acetobacter aceti* NCIB 8554 (our no. LBGB 4114) was used. The growth medium contained (per litre of a 50 mM-potassium phthalate buffer, pH 5.5); 0.5 g KH_2PO_4 , 0.5 g $\text{Na}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 2 g $(\text{NH}_4)_2\text{SO}_4$, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 10 mg NaCl and 10 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. Sodium acetate (30 mmol) was added as an organic substrate. The cultures were incubated for 15 h at 30 °C in flasks containing baffles on a rotary shaker or in a 12 l CHEMAP fermenter.

Cell-free extracts. At the end of the incubation, the cells were harvested by centrifugation (7000 g, 10 min, 4 °C) and washed in 0.2 M-saline. The pellet was resuspended in 0.1 M-Tris/HCl buffer, pH 7.4 (1 g wet weight in 10 ml buffer) and pressed through a French pressure cell at 40 MPa. Cell debris was removed by a 15 min centrifugation at 20000 g. The supernatant was called crude extract.

Protein determination. The protein content was determined by the Lowry method. For assay of intact cells, the method was modified (Herbert *et al.*, 1971).

Enzyme activities. Citrate synthase (EC 4.1.3.7) was determined by the method of Weitzman (1969). Particulate glucose dehydrogenase (EC 1.1.99.17) was assayed by the method of Wood *et al.* (1962) with phthalate buffer replacing McIlvain buffer.

Preparation of vesicles. Glycine (2.7 mM) and 10 mM-MgSO₄ were added to a culture of exponentially growing cells. After a further 15 h incubation, the cells were recovered by centrifugation and washed twice with a solution of 150 mM-NaCl and 10 mM-MgSO₄. This was followed by an osmotic shock using the method of Neu & Heppel (1965). The resulting sediment was resuspended in 0.1 M-potassium phosphate buffer, pH 7.4, with 10 mM-(NH₄)₂SO₄ as a stabilizer. Peptidoglycan degradation was initiated by the addition of 0.2 mg lysozyme ml⁻¹. After 8 min incubation, the resulting spheroplasts were stabilized with 20 mM-MgSO₄. Osmotic fragility was assayed by observation of the decrease of optical density at 546 nm after a tenfold dilution with water. For lysis of the spheroplasts and isolation of the vesicles, the methods of Kaback (1971) were followed. Unlysed cells and spheroplasts were removed by centrifugation at 200 g for 15 min. The supernatant membrane preparation was homogenized with the aid of a gas-tight Hamilton syringe equipped with a 22 gauge needle. The homogenized vesicle suspension in 0.1 M-potassium phosphate buffer, pH 6.6, with 20% (w/v) sucrose was layered on top of a solution of 60% (w/v) sucrose in the same buffer. After ultracentrifugation (64000 g, 100 min), the purified membrane layer was removed by aspiration, washed three times in 0.1 M-potassium phosphate buffer, pH 6.6, with 10 mM-EDTA and resuspended in the same buffer without EDTA. For storage the membrane preparation was frozen in batches in liquid nitrogen.

Uptake experiments in vesicles. These were done by the filtration method of Kaback (1974). The reaction volume was 50 µl containing 80 µg protein. The substrate was L-[U-¹⁴C]valine (9.25 MBq µmol⁻¹). Potassium phthalate buffer (50 mM), pH 6.6, with 0.1 M-LiCl was used as washing buffer.

Sample preparation for electron microscopy. Freeze-fractures were prepared by the method of Müller *et al.* (1980). For thin sections, the frozen samples were freeze-substituted by the method of Ebersold *et al.* (1981) and embedded in the plastic material K4M (Carlemalm *et al.*, 1982). A Philips electron microscope was used.

RESULTS AND DISCUSSION

Attempts to prepare vesicles by standard methods (Kaback, 1971) failed. We were unable to obtain spheroplasts either using the penicillin (Lederberg, 1956) or the lysozyme/EDTA method (Mahler & Fraser, 1956). It was therefore necessary to develop a special procedure. The method consisted of cultivation with the addition of glycine, osmotic shock and lysozyme digestion (for details, see Methods). The ensuing spheroplasts from lysozyme digestion are usually protected against lysis by a hypertonic solution of 0.3–0.5 M-sucrose (Kaback, 1971). This can, however, not be used with *A. aceti* since, for reasons which are not yet clearly understood, sucrose largely prevents lysozyme action. The same phenomenon was observed by Matsushita *et al.* (1981) with five different strains of acetic acid bacteria; their successful stabilizer was hypertonic saline. Bhandari & Nicholas (1980) used LiCl for *Nitrosomonas europaea*. We used $(\text{NH}_4)_2\text{SO}_4$ and MgSO_4 . EDTA had no effect on lysozyme digestion. For the final steps from spheroplasts to vesicles, standard methods could be efficiently used. The results of the procedure were checked with activity tests of marker enzymes and with electron micrographs of thin-section and freeze-fracture preparations.

Enzyme tests of a membrane vesicle preparation of acetate-grown cells are shown in Table 1. Citrate synthase served as a cytoplasmic marker and the quinoprotein glucose dehydrogenase as a membrane-bound marker. The specific activity of citrate synthase decreased during the procedure of vesicle preparation to about 1% of its original value. The specific activity of glucose dehydrogenase increased at the same time to about 400% of its original value. This indicates that about 25% of the protein of whole cells was incorporated in the membrane vesicles of *A. aceti*. The corresponding figure for membrane vesicles of *Escherichia coli* is 15% (Kaback, 1971).

In electron micrographs of thin sections of cells grown in the absence (Fig. 1*a*) and in the presence (Fig. 1*b*) of glycine both types of cells appeared intact. Only the dense layer of the cell envelope between the outer and the cytoplasmic membranes appeared to be dissolved in the cells grown in the presence of glycine (Fig. 1*b*). This illustrates the known mode of action of glycine, i.e. the replacement of L- and D-alanine during the synthesis of peptide subunits leading to a more loosely cross-linked peptidoglycan (Hammes *et al.*, 1973).

Electron micrographs of freeze-fracture preparations (Fig. 2) observed in the direction of shading (arrows) revealed typical differences between concave and convex fracture faces. The concave (inner) faces had a regular structural pattern whereas the convex (outer) faces were irregularly covered with warty protuberances. This was observed with both whole cell (Fig. 2*a*, *b*) and vesicle preparations (Fig. 2*c*) indicating that the vesicles were right side out. A comparison of Fig. 2(*a*) with Fig. 2(*b*) showed that the surface of normally grown cells appears smooth and that of cells grown with glycine appears wrinkled.

In an uptake experiment with the amino acid valine the competence of the vesicles for energy-dependent transport was tested. Valine uptake was increased threefold by the addition of

Table 1. *Activity of marker enzymes during the preparation of vesicles of A. aceti*

Specific activities of the cytoplasmic marker citrate synthase and the membrane marker glucose dehydrogenase on harvesting, after an osmotic shock and in the vesicles. Each test was done in quadruplicate.

Enzyme	Specific activity $\pm$ SD (nkat mg ⁻¹)		
	At harvest	After osmotic shock	In the vesicles
Citrate synthase	37 $\pm$ 4	36 $\pm$ 4	0.45 $\pm$ 0.03
Glucose dehydrogenase	0.26 $\pm$ 0.05	0.29 $\pm$ 0.06	1.07 $\pm$ 0.10

Fig. 1. Electron micrographs of *A. aceti* thin sections. (a) Whole cells grown without the addition of glycine; (b) whole cells grown with 2.7 mM-glycine; (c) a purified vesicle preparation. Bar, 0.2 μ m.

Fig. 2. Electron micrographs of *A. aceti* freeze-fractures. The arrows show the direction of shading. (a), (b) and (c) are as in Fig. 1. Bar, 0.2 μm .

Fig. 3. Influence of different substrates and of an uncoupler on L-valine uptake in vesicles of acetate-grown *A. aceti*. Membrane protein (80 µg) was suspended in 50 µl 0.1 M-potassium phosphate buffer, pH 6.5, containing 50 mM-MgSO₄ and subsequently incubated for 2 min at 30 °C. The experiments were started by the addition of 0.4 mM-L-[U-¹⁴C]valine (9.25 MBq µmol⁻¹). At appropriate times 1 ml 50 mM-phthalate buffer, pH 6.5, containing 0.1 M-LiCl was added. The suspension was mixed and filtered through a membrane filter. Filters were washed with 3 ml buffer and dried at 80 °C. The blanks were determined by adding radioactive substrate immediately before filtering the diluted vesicle suspension. Radioactivity was determined in a liquid scintillation counter. ●, No energy source; ▲, with 20 mM-DL-lactate; ■, with 20 mM-glucose; ○, with 20 mM-DL-lactate and 5 mM-2,4-dinitrophenol.

glucose or of DL-lactate (Fig. 3). This effect was abolished by the addition of the uncoupling agent 2,4-dinitrophenol.

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