The Enzymic Activity of the Outer Shell of *Lactobacillus arabinosus*

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SUMMARY

Preparations of the outer shell of *Lactobacillus arabinosus* were made by crushing the bacteria in a Hughes press and removing the cytoplasm by washing and centrifugation. Such preparations had none of the enzymes associated with flavin-mediated oxidation; they had consistently adenosine triphosphatase (ATPase) activity. The kinetics and other properties of this enzyme were studied. The evidence suggested that the enzyme was bound to the cytoplasmic membrane, the bulk of which appeared to be within the cell wall. The preparation was thus similar to that from obligate facultative aerobes and which, in contrast to the preparations from lactobacilli, contained enzymes associated with oxygen utilization mediated by the cytochrome hydrogen transport system. The bound ATPase had an optimum at pH 6.0 and was not markedly inhibited by ouabain or oligomycin: it was stimulated by 2,4-dinitrophenol. It was thus different from ATPase associated with ion transport but similar to that from mitochondrial membranes. There was no nucleotide pyrophosphorylase activity in the preparation but other nucleotide triphosphates and diphosphates and some inorganic phosphates were hydrolysed but at slower rates than was ATP.

INTRODUCTION

In previous work (Hughes, 1962; Francis, Hughes, Kornberg & Phizackerly, 1963) it was shown that the outer shells of many aerobic bacteria may be prepared by crushing the organisms in a Hughes (1951) press and removing the cytoplasmic contents by washing and centrifugation. Such preparations have been called cell-wall membranes because they have the form and chemical constituents of the cell wall and also the phospholipid components and enzymic activities associated with the cytoplasmic membrane. In the present work similar preparations were made by the same method from *Lactobacillus arabinosus*. This organism normally does not use oxygen as a final hydrogen acceptor for energy (ATP) production but can utilize oxygen to oxidize several substrates by flavin enzymes which were thought to be soluble; the exception was the pyruvate oxidase which was particulate (Strittmatter, 1959). However, the enzyme preparations from *L. arabinosus* were made by prolonged ultrasonic treatment, a process which comminutes the outer shells of bacteria into fine fragments and solubilizes some enzymes associated with them. The cell-wall membranes were found not to contain these flavin enzymes which were all in the soluble fractions, but did contain an adenosine triphosphatase

(ATPase). The properties of this enzyme have now been investigated. The evidence presented here supports the view that the ATPase is bound to the cytoplasmic membrane. While this work was in progress an ATPase has been found in similar preparations from the obligate anaerobes *Clostridium welchii* and *C. sporogenes*. It was also present in membrane preparations 'ghosts' from spheroplasts of this latter organism (Galli & Hughes, 1965). The enzyme is also present in membrane preparations and cell-wall membranes of aerobes. So far it has not been possible to assign a role to this membrane-bound bacterial ATPase.

METHODS

Growth of Lactobacillus arabinosus. *L. arabinosus* strain 17-5 was grown in a medium (Barton-Wright, 1952) containing amino acids, B-group vitamins, glucose and salts. In 1 l.; 20 g. Oxoid tryptone; 6 g. Oxoid yeast extract; 20 g. glucose; 10 g. sodium acetate; 4.5 g. KH_2PO_4 ; 26 ml. N-NaOH; 5 ml. essential salts solution; 4 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.1 g. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$; 0.04 g. anhydrous FeCl_3 in 100 ml. dilute hydrochloric acid; adjusted to pH 7.6. Stock cultures of the organism were sub-cultured every 2 weeks, grown at 30° and stored at 2°.

Large quantities of the organism were grown in 10 l. bottles nearly full of medium which were inoculated with a growing culture. After incubation for 18–20 hr, when the medium had become about pH 4.5, the bacteria were collected with a Sharples continuous centrifuge. The bacteria were then washed well with 0.1 M-tris + maleate buffer (pH 7), centrifuged again and resuspended in a small volume of this buffer. The average yield was 7 g. wet wt. bacteria/l. medium.

Preparation of cell-wall membranes. In a typical preparation, about 60 g. wet-weight bacteria, suspended in a small volume of 0.1 M-tris + maleate buffer (pH 7), were crushed at -25° in a Hughes (1951) press. The material obtained was thawed, suspended in 2 vol. of a mixture of equal volumes of 0.1 M-tris + maleate buffer (pH 7) and 0.9 % NaCl in a loose-fitting glass homogenizer and incubated for 30 min. at about 2° with 100 µg. deoxyribonuclease to decrease the high viscosity of the preparation and facilitate emptying of the broken bacteria. The degree of breakage was judged at this stage by electron microscopy (Pl. 1, fig. 1). The preparation was then centrifuged at 30,000 g at 2° for 30 min. and separated into a supernatant fraction and a solid residue. The residue consisted of two layers, the uppermost being composed of cell-wall membranes (CWM). This layer was removed by using a curved spatula and resuspended in buffer saline mixture and centrifuged at 30,000 g for 30 min. twice more. The material thus obtained was usually equivalent to about 1–3 g. dry wt. This material was resuspended in about 75 ml. of 0.1 M-tris + maleate buffer (pH 7) and stored at 2°. The preparation then consisted of electron-transparent hulls of bacteria, containing very little electron-dense material (Pl. 1, fig. 2).

An attempt was made to produce cell-wall membranes by using a French press (Milner, Lawrence & French, 1950) in which a 20 % (v/v) suspension of bacteria in buffer at 4° was broken by liquid shear at pressures of 15×10^3 lb./sq.in. Electron microscopy showed that a suspension had to be passed through the French press at least six times to produce the same degree of breakage as was produced by one treatment in the Hughes press. This resulted in further comminution of the cell-wall membranes and so the method was not used further.

Further fragmentation of the cell-wall membranes by ultrasonic treatment. The suspension (25 ml.) of washed cell-wall membranes (about 0.2 g. wet wt./ml.) in 0.1 M-tris + maleate buffer in a 100 ml. beaker cooled in ice was disrupted by ultrasonic treatment with a 600 W. Mullard magnetostrictor oscillator operating at a power level 6.9 V. at a setting of 65/100 for 45 min. Electron microscopy showed that the cell-wall membranes after this treatment were considerably fragmented (Pl. 1, fig. 3). The solid fragments were removed by centrifugation at 100,000 g for 90 min. at 0° in a Spinco model L preparative ultracentrifuge. The clear supernatant fluid was separated from the residue and the latter resuspended in 25 ml. of the original buffer.

Electron microscopy. Cell fractions for routine work were dried on to Formvar-covered grids and examined by transmission. To gain a more accurate knowledge of the shape of the particles in the fractions, the samples were fixed with 1 % osmic acid and then shadowed with 40 % Au + 60 % Pd. All material was examined in an Akashi TR 50 Tronoscope at initial magnifications between 600 and 15,000.

Assays of adenosine triphosphatase activity. It was assumed that the enzyme hydrolysed ATP according to the equation: $\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{H}_3\text{PO}_4$. Thus assays for either the phosphate ion or the first highly acidic hydrogen ion of H_3PO_4 both estimated the activity of the enzyme.

Assay of phosphate release. In most experiments about 15 mg. wet weight solid or 0.1 ml. of supernatant fluid were incubated at 37° with ATP (0.3–20 mM); MgCl_2 at the same concentration as that of ATP; 0.5 ml. 0.1 M-tris + maleate buffer (pH 7.0); in total volume of 1–2 ml. Incubation times varied between 15 min. and 2 hr. After incubation the tubes were cooled to 2° and 1 ml. of cold 5 % (w/v) perchloric acid added. Tubes were then centrifuged at about 5000 g in cooled centrifuge cups to remove precipitated protein. Duplicate samples of the supernatant fluid were added to 8 ml. water in 10 ml. graduated tubes and the inorganic phosphate estimated by the method of Fiske & SubbaRow (1925). The reducing agent was 1-amino-2-naphthol-4-sulphonic acid, sodium sulphite and bisulphite. A blank without reagents and a standard containing 1 μ mole orthophosphate were also treated with the reagents. E_{700} was read in glass cuvettes in a Unicam S.P.600 spectrophotometer. The amount of phosphate was calculated from standard curves and expressed as a mean of the two samples from which the inorganic phosphate of ATP and the enzyme was deducted (< 0.2 μ mole).

Assay of hydrogen-ion release. Continuous titrations were carried out at 37° with NaOH in a Radiometer automatic titrator, with a combined calomel and hydrogen electrode. The initial volume of cell-wall membrane and MgCl_2 was 1 ml.; no buffer was added to the incubation mixture, which was kept constant at pH 7.4. This is slightly higher than the optimum pH value, but consistent results were not obtained at pH 6.0.

Typical mixtures contained 0.4 mM- MgCl_2 ; 50 mg. wet weight cell-wall membrane, in a total volume of 1 ml. This mixture was at about pH 6; 0.1 N-NaOH was added to bring to pH 7.4. The mixture was kept at 37° and stirred with CO_2 -free air. Various volumes of 0.2 M-ATP were then added to start the reaction and the microburette added sufficient 0.01 N-NaOH to maintain the mixture at pH 7.4. The volume of NaOH added was recorded as a function of time; the slope of this curve was therefore proportional to the rate of reaction.

Chemical analysis of cell fractions. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with the Folin-Ciocalteu reagent and measurement of the extinction (E_{625}) of the blue colour in a Unicam S.P. 600 spectrophotometer. For carbohydrate analysis, fractions were hydrolysed by 4 N-sulphuric acid at 100° for 2 hr in a sealed tube, after which insoluble material was removed by centrifugation at 5000 g. In this supernatant fluid, total carbohydrate was estimated by the method of Dische (1929); methylpentoses by the method of Dische & Shettles (1948) and glucosamine by a modified method of Aminoff, Morgan & Watkins (1951), Levvy & McAllen (1959). Deoxyribonucleic acid in the original samples was estimated by the method of Burton (1956) and ribonucleic acid in the same samples by the method of Mejbaum (1939).

Lipid was extracted with a 2 + 1 mixture of redistilled chloroform + methanol and the extract washed twice with 0.5 vol. of water. After drying the chloroform + methanol extract with anhydrous sodium sulphate, the extract was evaporated under nitrogen and then under vacuum to dryness in weighed beakers by an infra-red lamp. Samples of this anhydrous material were then saponified with methanolic (50 %) 2 N-potassium hydroxide solution and esterified in light petroleum (40–60°) with diazomethane in ether, then applied to a gas chromatography column, as described below.

Chromatographic methods. Nucleotides and inorganic phosphate were separated by paper chromatography by the method of Krebs & Hems (1953).

For the chromatography of sugars, samples of carbohydrate, hydrolysed with H_2SO_4 , were neutralized with saturated baryta, the barium sulphate was removed by centrifugation and the solutions evaporated to 0.5 ml. under a Magda infra-red lamp. The solvents used were phenol + water and *n*-butanol + acetic acid + water (Woiwod, 1949). A standard sugar sample was placed on each chromatogram (Putman, 1957) *m*-phenylenediamine hydrochloride was used as the developing agent (Chernick, Chaikoff & Abraham, 1951). The relative R_F values used were those of Partridge & Westall (1948).

Methyl esters of fatty acids were separated by gas chromatography in an Argon Chromatograph (Pye Co. Ltd., Cambridge). The support was acid and alkali-washed Celite 535 (Johns-Manville Co. Ltd., London, S.W. 1). Apiezon L high-vacuum grease (10 %) was the non-polar stationary phase. Samples (0.1 μ l.) of dry ester were placed on the column by the method of Renshaw & Biran (1962). Peak areas were determined by triangulation according to Kouleman (1957). The chromatographic peaks were identified by comparison of the retention volumes with known acids and by hydrogenation and chromatography of the products.

The measurement of radioactive phosphorus. ^{32}P in solution was estimated in a conventional Geiger-Miller 10 ml. counting tube type M6 (supplied by 20th Century Electronics Ltd., Croydon, Surrey), operating at 1090 V. in a lead castle from E.R.D. Engineering Co. Ltd., Slough, Middlesex. The scaling unit was type N 530 F obtained from Ekco Electronics Ltd., Southend-on-Sea, Essex.

For the measurement of radioactivity on paper chromatographs, an end-window counter operating at 1625 V. attached to a Panex Scaling Unit type D 657 was used. Radioactive phosphorus on chromatograms was measured by shielding the spot with a lead plate 2 mm. thick with a hole 2 cm. + 3 cm. in the centre.

Chemicals. ^{32}P -labelled adenosine triphosphate was made by the method of

Glynn & Chappell (1964). Starting with 1 mc of orthophosphate, the final activity of the terminal phosphate group of the ATP was 300 μ c; very little activity appeared in the β -phosphate group. Chromatograms indicated that some labelled AD³²P was also present.

All other chemicals used were commercial preparations and were not purified unless so stated.

RESULTS

The rate of hydrolysis of ATP by the various preparations from *Lactobacillus arabinosus*, when measured by the formation of orthophosphate or hydrogen ions, proceeded linearly until about half the ATP was hydrolysed; the rate then decreased gradually (Fig. 1). The release of phosphate from increasing amounts of ATP

Fig. 1

Fig. 2

Fig. 1. Reaction curves of ATPase measured by phosphate release and by titrating the H⁺ formed. The cell-wall membrane preparation was incubated with ATP (10 μ mole) and phosphate released and H⁺ formation measured as described in the text. ●—●, Phosphate release; —, H⁺ formation.

Fig. 2. The effect of ATP concentration on phosphate release by the ATPase of cell-wall membrane preparations from *Lactobacillus arabinosus* 17-5. A cell-wall membrane preparation was incubated with various concentrations of ATP and the phosphate released estimated after incubation for 30 min.

with constant amounts of cell-wall membrane was measured under standard conditions. Consistent results were obtained only when the molar concentration of Mg²⁺ was the same as that of ATP. When the concentrations of ATP and Mg²⁺ were equal, plots of phosphate release against ATP concentration increased to a maximum and then decreased (Fig. 2). This suggested that ATP at high concentration was inhibitory. The concentration of ATP needed to produce the maximum rate of hydrolysis varied with different batches of cell-wall membranes and was not proportional to their specific activity. Lineweaver-Burk plots gave an approximate value of K_m of 3 mM and of V of 1.7×10^5 μ mole P/g. dry wt./hr. at low values of substrate concentration. Similar results were obtained when the hydrogen ion formation was measured. The rate of the initial reaction then was plotted as a function at the

concentration of ATP and gave a K_m of 6–12 mM. The ATPase showed a marked optimum at pH 6·0 when measured in tris + maleate buffer (Table 1).

Hydrolysis of compounds other than ATP. Suspensions of cell-wall membrane hydrolysed ADP at 20–30 % of the rate of that of low concentrations of ATP. No maximum or minimum was observed in the curve of phosphate release against concentrations of ADP up to 16 mM (Table 2). The value of K_m was 12 mM and V was 1×10^4 μ mole P/g. dry wt. cell-wall membrane/hr. To test that the same enzyme was acting on the two adenine nucleotides, equal concentrations of ATP and ADP alone and mixed were incubated with cell-wall membrane. The release of phosphate was not additive; the release from ATP with ADP was less than with ADP alone. This suggests that the same enzyme hydrolyses both ATP and ADP.

Table 1. *Variation with pH value of ATPase activity of cell-wall membrane preparations from Lactobacillus arabinosus 17–5*

Tubes contained 0·4 ml. tris + maleate buffer (pH 7); 0·1 ml. 50 μ mole ATP/ml.; 0·1 ml. 50 μ mole $MgCl_2$ /ml.; cell-wall membrane (equiv. 15 mg. dry wt.). Measured amounts of 0·2M-acetic acid or sodium hydroxide were added to bring the mixtures to between pH 4·6 and 10 as measured on a pH meter. The pH value of control tubes without enzyme or ATP was adjusted similarly. The tubes were then incubated at 37° for 20 min. and phosphate was determined as described previously.

pH value of mixture before incubation	Phosphate release (μ mole P/g. dry wt. cell-wall membranes/hr.)
4·6	880
5·1	2080
5·6	2460
6·2	2500
6·8	2300
7·5	1690
7·8	1870
8·4	1750
9·0	1380
10	1300

Table 2. *The effect of ADP concentration on the rate of hydrolysis of adenosine diphosphate by cell-wall membranes*

ADP at various concentrations was incubated under the usual conditions and phosphate release estimated as described in the text.

ADP concentration (mM)	Phosphate release (μ mole P/g. dry wt. cell-wall membranes)
4·0	180
6·0	300
8·0	350
9·0	470
12·0	590
13·5	980

To estimate the effect of released ADP on the value of K_m found for the hydrolysis of ATP, various amounts of ATP were incubated with cell-wall membrane for various times up to 40 min. The value of K_m for each time was found by a Lineweaver–Burk plot. The straight lines produced in each case all cut the x -axis at the same point, giving a value of K_m of about 10 mM. Thus increasing concentrations of

ADP produced by the hydrolysis of ATP had no appreciable effect on the value of K_m found by this method.

Suspensions of cell-wall membrane had no effect when incubated with adenosine monophosphate, other nucleoside diphosphates, sugar phosphates and NAD, at the times and concentrations normally used with ATP. All the nucleoside triphosphates tested were hydrolysed at a slow rate by the cell-wall membrane. Inosine triphosphate was the most rapidly attacked: the rate of hydrolysis was 50 % of that of ATP under the same conditions (Table 3).

Table 3. *The hydrolysis of nucleotide phosphates by cell-wall membrane preparations from Lactobacillus arabinosus 17-5*

Tubes contained 0.47 and 1.41 mM nucleotides. Comparison tubes held similar concentrations of ATP and ADP, and a similar concentration of Mg^{2+} ; 20 mg. wet wt. cell-wall membrane preparation; 0.5 ml. 0.1 M-tris + maleate buffer (pH 7); total volume 1.2 ml. The tubes were incubated at 37° for 1 hr and phosphate was determined in the usual way, in duplicate. The amount of phosphate released is expressed after the deduction of blanks without cell-wall membrane or nucleotide.

Nucleotide	Phosphate release*	
	Nucleotide conc. (0.47 mM)	Nucleotide conc. (1.41 mM)
Adenosine monophosphate	0	0
Adenosine diphosphate	27	32
Cytidine diphosphate	0	0
Inosine diphosphate	0	0
Uridine diphosphate	0	0
Guanosine diphosphate	0	0
Cytidine triphosphate	18	15
Inosine triphosphate	42	57
Guanosine triphosphate	32	46
Thymidine triphosphate	40	26
Uridine triphosphate	40	45

* Expressed as $\frac{\text{Phosphate release from nucleotide}}{\text{Phosphate release from ATP under identical conditions}} \times 100 \%$.

The cell-wall membrane preparations also hydrolysed condensed inorganic phosphates. Pyrophosphate was hydrolysed at the same rate as ATP under the same conditions; tripolyphosphate and tetrametaphosphate were hydrolysed slowly; hexametaphosphate was attacked at significant rates (Table 4).

The reversibility of the reaction was tested by incubating the cell-wall membranes with ATP and ADP in the presence of P_i or $AT^{32}P$. The nucleotides and inorganic phosphate were separated by paper chromatography and the activity of each was found. When the cell-wall membrane was incubated with inorganic radioactive orthophosphate, only the phosphate spot was labelled on the chromatogram; no exchange had taken place. There was no increase of activity in the ADP spot when cell-wall membrane preparations were incubated with $AT^{32}P$; the bulk of the activity appeared in the orthophosphate. There was thus no indication of reversibility of the hydrolysis of ATP by these cell-wall membrane preparations.

$AT^{32}P$ was also incubated with other nucleotides and their activity measured after separation by paper chromatography. Consistent labelling was found in uridine triphosphate and cytidine triphosphate only. The amount of ^{32}P incorporated

represented only 1 % of that added as AT^{32}P but the specific activity, although not measured, appeared to be high.

Inhibition of the ATPase. 2,4-Dinitrophenol decreased the inhibition of the rate of hydrolysis by excess ATP and at high concentrations of DNP (4 mM) appeared to activate the ATPase. Chloramphenicol had a similar effect. There was no apparent stimulation at concentrations of ATP which did not inhibit.

Table 4. *The hydrolysis of inorganic phosphate anhydrides by cell-wall membrane preparations from Lactobacillus arabinosus 17-5*

Tubes contained various amounts of the phosphate anhydrides or of ATP and MgCl_2 at the same concentrations; about 20 mg. wet wt. of cell-wall membrane. At the two highest concentrations of ATP, 0.5 ml. of tris maleate+buffer (pH 7) was in a total volume of 1.5 ml.; at the other concentrations of ATP, total volume was 1 ml. Where the time and concentration of ATP is the same as that of a phosphate compound, the two incubations were carried out at the same time. Controls for the phosphate content of the compounds and the cell-wall membrane have been deducted as usual.

Substance	Final concentration ($\mu\text{mole/ml.}$)	Time of incubation (hr)	Release ($\mu\text{mole P/g. dry wt./hr}$)	Hydrolysis of phosphate compound (%)
ATP	2	0.33	1480	62
	2	1	250	55
	2	2	240	107
	3.75	1	735	65
	15	1	2570	57
$\text{Na}_4\text{P}_2\text{O}_7$	2	0.33	623	13
	2	1	477	53
	2	2	261	58
	3.75	1	855	42
	15	1	113	1
$\text{Na}_5\text{P}_3\text{O}_{10}$	2	0.33	0	0
	2	1	7.2	16
	2	2	5.0	22
$(\text{NaPO}_3)_4$	2	0.33	0	0
	2	1	10.8	18
	2	2	3.0	13
$(\text{NaPO}_3)_6$	2	0.33	0	0
	2	1	527	117
	2	2	362	181

Oligomycin and ouabain had no effect on the hydrolysis of ATP by the cell-wall membrane at 10 $\mu\text{g. dry wt./ml.}$ These concentrations were sufficient to inhibit the Na^+ stimulated and K^+ stimulated brain ATPases almost completely.

The effects of the sulphydryl-blocking reagents phenylmercuriacetate and *p*-chloromercuribenzoate were complicated. Further inhibition was found at some concentrations of ATP which were alone sufficient to cause inhibition of the ATPase. At a higher concentration of ATP there was apparent stimulation (Table 5). Sodium deoxycholate had similar effects to those of sulphydryl-blocking agents. Hexadecyltrimethylammonium bromide (Cetavlon) had no effect on the ATPase at or below 0.1 mM. At 1 mM, that is, above the critical point of micelle formation, the phosphate release decreased to 10–20 % of its normal value. This inhibitory concentration was a hundred times that required to prevent growth of the *Lactobacillus arabinosus* strain used. Ammonium sulphate had an inhibitory effect at high concentrations. Fluoride (5 mM) inhibited the enzyme strongly over all concentrations of ATP tested.

Ultrasonic treatment of the cell-wall membrane. Treatment of suspensions of cell-wall membrane preparations in the MSE Mullard cell disintegrator released various amounts of ATPase and comminuted the membranes into increasingly finer fragments (Pl. 1, fig. 3), depending on the time of treatment. After centrifugation at 100,000 *g* for 2 hr of a preparation treated ultrasonically for 45 min. the supernatant fluid, although clear, was enzymically active (Table 6). The solid deposit and the supernatant fluid both showed about a threefold increase in their maximum

Table 5. *The effect of phenylmercuriacetate (PMA) and p-chloromercuribenzoate (CMB) on phosphate release from ATP by cell-wall membrane preparations*

Tubes contained various amounts of ATP; cell-wall membrane (15 mg. wet wt.); Mg^{2+} at the same concentration as ATP; 0.5 ml. 0.1 M tris + maleate buffer (pH 7); 0.75 mmole mercury compound in a final volume of 1.5 ml. The tubes were incubated at 37° for 45 min., after which the phosphate release was estimated in the usual way.

	ATP conc. (mM)		
	(3.3)	(6.7)	(13)
	P release (μ mole P/g. dry wt. cell-wall membrane/hr.)		
ATP alone	2760	4120	760
ATP + PMA	2140	3600	2350
ATP + CMB	2020	3540	3260

Table 6. *The effect of various concentrations of ATP on ultrasonically treated cell-wall membrane of Lactobacillus arabinosus 17-5 and its supernatant fluid*

Tubes contained different concentrations of ATP; ultrasonically treated cell-wall membrane (20 mg. wet wt.) or the corresponding 100,000 *g* supernatant fluid (equiv. 4 mg. dry wt.); Mg^{2+} at the same concentration as ATP; 0.5 ml. of 0.1 M-tris + maleate buffer (pH 7) in a total volume of 1.5 ml. After incubation for 30 min. at 37° phosphate release was estimated in the usual way.

Conc. of ATP (mM)	Phosphate release by μ mole P/g. dry wt./hr.	
	ultrasonically treated cell-wall membranes	100,000 <i>g</i> supernatant fluid
3.3	14,700	15,100
6.7	20,600	16,900
13.3	2380	1110

specific activity. The value of K_m for the supernatant fluid was 0.6 mM. In other properties the ATPase in the supernatant fluid appeared very similar to that in the untreated cell-wall membrane preparation. This included the inhibitory effects of high concentrations of ATP, and the effect of 2,4-dinitrophenol. Further centrifugation of this supernatant fluid for 16 hr at 100,000 *g* did not yield a solid pellet, nor distinct layering. The supernatant fluid was separated into upper, middle and bottom fractions. The bottom fraction was considerably enriched in both protein and enzymic activity (Table 7). The addition of ammonium sulphate up to 80 % saturation of the clear 100,000 *g* supernatant fluid precipitated the bulk of the enzyme activity. The first precipitate, up to 50 % saturation with ammonium sulphate would not redissolve in water or various buffers; the other fractions did resuspend to give clear liquids. There was no increase in specific activity in any fraction obtained by this treatment.

The effect of enzymes on ATPase activity. Cell-wall membrane preparations were incubated with various hydrolytic enzymes at values between pH 5 and 8. After incubation the particulate material was removed by centrifugation and the ATPase of this deposit and the supernatant fluid compared with controls incubated without the addition of enzymes (Table 8). In most cases there was a marked decrease of ATPase activity, except in the case of material digested with pancreatic lipase where there was a marked increase in activity in the supernatant fluid but no decrease of

Table 7. *The protein and ATPase distribution after the long centrifugation of the 100,000 g supernatant fluid of the ultrasonically treated cell-wall membranes of Lactobacillus arabinosus 7-5*

Protein determination was by the usual method. For the ATPase determination, tubes contained ATP and Mg^{2+} (8.4 mM); a fraction of the supernatant fluid (0.2 ml.); 0.5 ml. of 0.1 M-tris + maleate buffer (pH 7) in a total volume of 1.3 ml. After incubation for 3 min. at 37°, phosphate release was estimated in the usual way. The figures are not corrected for the negligible phosphate content of the supernatant fractions.

Layer of supernatant fluid	Phosphate release (μ mole P/ml./hr)	Protein content (mg./ml.)
Lower	19.6	16.5
Middle	1.8	4.5
Upper	1.3	4.3

Table 8. *The effect on the cell-wall membrane preparations from Lactobacillus arabinosus 17-5 of incubation with various enzymes*

Cell-wall membranes (50 mg. wet wt.) were incubated with about 1 mg. of the enzymes shown for 1.5 hr at 37° in buffer of the shown pH value. After centrifugation at 10,000 g for 5 min., the supernatant fluid was separated from the solid cell-wall membrane and both fractions were tested for ATPase activity. A control tube was incubated containing no external enzyme at pH 7. These fractions were then incubated with 3 mM ATP and Mg^{2+} and 0.5 ml. 0.1 M-tris + maleate buffer (pH 7) in a total volume of 1 ml., for 45 min. at 37°. Phosphate release was then estimated in the usual way. Blanks were run as usual for the phosphate content of the ATP and the incubated fractions. Phosphate release is expressed as a percentage of the untreated control.

Enzyme	pH value of incubation mixture	Relative phosphate release (%)	
		Supernatant fraction	Cell-wall membrane fraction
Papain	6	14	49
Hyaluronidase	5	19	30
Mycozyme	7	38	44
Lysozyme	7	94	107
Trypsin	8	11	14
Rattlesnake venom	7	51	33
Phospholipase D + Ca^{2+}	8	—	62
Lipase (pancreatic)	8	510	92

activity in the solid deposit. It is possible that this result indicates a release of ATPase activity accompanied by an activation of the released enzyme. As with the preparations partially solubilized by ultrasonic treatment, it was not found possible to fractionate further the enzyme released by lipase by the usual precipitation methods.

Chemical composition of cell-wall membrane. The cell-wall membranes contained

20–23 % protein compared to 27–30 % present in the whole bacteria (calculated on dry wt.). The total lipid varied from 7 to 11 % and represented the bulk of that contained in the bacteria; that remaining in the supernatant fluid was separable by further centrifugation. The lipid after extraction contained about 4 % phosphorus and can thus be assumed to be mainly phospholipid. Methyl esters of the fatty acids were prepared after saponification and examined by gas chromatography. There was no significant difference in the carbon number of the fatty acids from whole bacteria, cell-wall membrane or the supernatant fluid fractions. The bulk of the acids had carbon numbers between C_{15} and C_{18} , but were not examined in detail. The total carbohydrate content of the cell-wall membranes was 37 % compared to 14 % of the whole bacteria. The glucosamine determinations in whole bacteria were not very consistent, but the carbohydrate of the supernatant fluid contained about 0.8 % glucosamine while that of the cell-wall membranes contained 2.4 %. Both supernatant fluid and cell-wall membranes contained considerable amounts of rhamnose which is often used as an indication of the presence of bacterial cell-walls; cell-wall membranes contained 9–12 % rhamnose. The DNA content of the cell-wall membranes was usually negligible, but some batches contained up to 0.8 %; this amount when present was usually associated with electron-dense granules trapped in relatively unfragmented cell-wall membranes. The RNA content of the cell-wall membranes was 7 % when DNA was mostly absent, but increased when the DNA content was higher and was presumably due to the trapping of cytoplasmic contents. The chemical analysis of fragments of cell-wall membrane isolated after ultrasonic treatment was substantially the same as that of the intact cell-wall membranes. A preliminary examination by paper chromatography of the amino acids obtained by acid hydrolysis of cell-wall membrane showed the complete range of typical proteins. The chemical analysis therefore confirms the idea that a large part of the cytoplasmic membrane is present together with the cell wall in these preparations.

Cellular distribution of enzymes other than ATPase. Supernatant and cell-wall membrane fractions were examined in some detail for the presence of the riboflavin-mediated oxidation of pyruvate, lactate and glycerophosphate, glucose and glycerol (Strittmatter, 1959). Oxygen uptake with these substrates by the fractions was measured manometrically in the Warburg apparatus, with and without methylene blue and riboflavin. In all cases the enzymic activity was in the supernatant fractions and the cell-wall membrane was without significant activity. The activity in the supernatant fractions was not removed by centrifugation at 25,000 *g* for periods up to 2 hr. Similar results were obtained with supernatant fractions from bacteria broken in a 'sonomec' shaker (Rodgers & Hughes, 1960) or by ultrasonic treatment for 15 min. There was some pyruvate oxidase in the solid fractions from bacteria treated ultrasonically for longer periods (up to 60 min). This is similar to the findings of Strittmatter who disintegrated bacteria in the Raytheon disintegrator for 60 min., and was possibly due to denaturation of soluble protein (Hughes, 1961). Other enzymes found in the supernatant fraction and not in the cell-wall membranes were hexokinase, myokinase and malic enzyme. Enzymes located in the supernatant fractions in previous work include nicotinamide deamidase (Hughes & Williamson, 1953), pantothenate and pantotheine phosphorylase (Pierpoint, Hughes, Baddiley & Mathias, 1955), cytidine pyrophosphorylase (Shaw 1961), lactic dehydrogenase (Don & Kaplan, 1960). It is also likely that most, if

not all, of the glycolytic enzyme system is soluble (Hughes, 1951). None of the cell-wall membrane preparations had polynucleotide phosphorylase activity when tested by the method of Abrams & McNamara (1962).

DISCUSSION

The outer shell or hull of *Lactobacillus arabinosus* strain 17-5 has been prepared by crushing the bacteria in the Hughes press and removing the cytoplasmic contents by washing. Such preparations formed inorganic phosphate from ATP at rates of 1.0-6 mmole P/g. dry wt./hr. Phosphate was also released from ADP at slower rates and kinetic evidence suggests that the same enzyme was involved in both reactions. There was also a phosphate release from various inorganic phosphates but it was not certain whether this too was due to the nucleotide phosphatase which hydrolysed ATP and ADP as follows: $\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{H}_3\text{PO}_4$. It is noteworthy that so far this is the only enzymic activity definitely associated with outer shells of *L. arabinosus*, whereas similar preparations from obligate and facultative aerobes contain a wide range of enzymes associated with energy production and electron transport (Hughes, 1962). The cell-wall membranes from a wide range of aerobically grown bacteria have also been found to have an ATPase similar to that reported here, and the enzyme has also been found in membrane preparations from *Streptococcus faecalis* (Abrams, McNamara & Johnson, 1960) and in preparations from *L. casei* supplied to us by Dr J. Shockman. More recently the enzyme has been found in cell-wall membranes and empty spheroplasts ('ghosts') of *Clostridium sporogenes* (Galli & Hughes, 1965) and in particulate fractions of *Chlorobium thiosulphatophilum* (Cole & Hughes, 1965). The hull preparations from *L. arabinosus* contained from 7 to 11 % total lipid of which the bulk appeared to be phospholipid since it contained about 4 % phosphorus. Most of the fatty acids previously reported to be present in this organism (Hofman, Lucas & Sax, 1952) appeared to be present in this hull lipid. A variable amount of ATPase activity was found in the supernatant fractions after centrifugal deposition of 'hulls', but the bulk of this could be recovered as a solid pellet by more prolonged centrifugation. This pellet also contained lipid and carbohydrate in about the same proportion as in the 'hulls' and it is assumed to be the result of their further comminution during disintegration; similar fragments were obtained by ultrasonic treatment. Up to now no satisfactory method has been found for solubilizing the ATPase of these hulls or of markedly increasing its specific activity. Of a number of enzymes, only pancreatic lipase brought about a release of some part of the enzymic activity from the particulate preparations, but this released enzyme could be regarded as insoluble since it was concentrated by centrifugation. The evidence therefore strongly supports the idea that the ATPase is bound to the protoplasmic membrane and that the membrane or large parts of it are associated with the wall in these preparations. They have therefore been assumed to be similar to cell-wall membranes previously described (Hughes, 1962). In the whole bacteria the ATPase cannot utilize exogenous ATP, presumably because the latter cannot enter.

Membrane bound ATPases are widely distributed in nature (Novikoff, Essner, Goldfischer & Heus, 1962) and are associated with ion transport (Whittam, 1964) changes in conformation (Kielly, Kimura & Cooke, 1964) and energy production

(Jagendorf, Hind & Neuman, 1964). The ATPase from *Lactobacillus arabinosus* differs from those associated with ion transport in that it is not markedly inhibited by ouabain and oligomycin; in experiments not reported here it was not markedly affected by Na^+ or K^+ . Its pH optimum was also markedly lower (pH 6.0) than ATPases associated with ion transport. On the other hand it is stimulated by DNP, as is the ATPase of fragmented mitochondrial cristae associated with oxidative phosphorylation.

Experiments with labelled ATP and lactobacillus ATPase preparations did not transfer terminal phosphate to any acceptor added, but the possibility cannot be ruled out that the ATPase is concerned in a phosphorylation associated either with cell-membrane or cell-wall synthesis. The lactobacillus preparations had no nucleotide pyrophosphorylase activity such as reported for membranes of *Streptococcus faecalis* (Abrams & McNamara, 1962). The consistent presence of RNA in the preparations of lactobacillus cell-wall membranes however lends support to the many suggestions that some membranes in bacteria play a role in protein synthesis in association with ribosomes or ribosomal RNA (Hunter, Brookes, Crathorn & Butler, 1959; Nisman & Fukuhara, 1961). It is possible that this ubiquitous ATPase is also involved. Up to the present however no definite role can be ascribed to this enzyme.

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Fig. 1

Fig. 2

Fig. 3

EXPLANATION OF PLATE

Fig. 1. Disintegrated *Lactobacillus arabinosus* 17-5 before fractionation by centrifugation. Bacteria were disintegrated by crushing in the Hughes press and treated with DNAase, fixed and mounted for examination without further treatment. $\times 6500$.

Fig. 2. Cell-wall membranes from *Lactobacillus arabinosus* 17-5 after purification. The disintegrated bacteria shown in fig. 1 were fractionated by centrifugation as described in the text. $\times 6500$.

Fig. 3. Cell-wall membranes of *Lactobacillus arabinosus* 17-5 after ultrasonic treatment. A purified preparation of cell-wall membranes was treated for 10 min. in the M.S.E. cell disintegrator, fixed and mounted as before. $\times 8500$.