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Studies of Controlled Lysis of Washed Cell Suspensions of *Lactobacillus fermenti* and Preparation of Membrane-like Fragments by a Combined Trypsin-Lysozyme Treatment

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The influence of lysozyme on washed cell suspensions of *Lactobacillus fermenti* was studied using different conditions.

Although the cells were not affected by lysozyme directly, nor after pre-treatment with heat, butanol, EDTA or urea, the addition of trypsin in advance or simultaneously rendered the cells susceptible to the action of lysozyme.

After lysis by a combined trypsin-lysozyme treatment, membrane-like fragments were isolated by differential centrifugation and were shown to exhibit ATPase activity.

It is now generally accepted that lysozyme (endoacetylmuramidase, *N*-acetylmuramide glucanohydrolase, EC 3.2.1.17), in addition to its action on chitin, also specifically hydrolyzes the β-1,4-glucosidic linkage in the repeating unit of the characteristic cell wall substance murein (peptidoglucan),* a newly discovered type of macromolecule, hitherto encountered only in the cell walls of bacteria and bluegreen algae.

Gram-positive bacteria with their high content of murein and low content of lipoprotein and lipopolysaccharide in the cell envelope (cell wall) are usually attacked by lysozyme whereas Gram-negative bacteria are as a rule resistant to a direct attack. This resistance is attributed to the more complex structure of their cell envelope consisting of a "double membrane" with the murein component embedded in it. However, varying degrees of sensitivity towards lysozyme seem to occur even among the Gram-positive bacteria, the cell walls of which often contain substantial amounts of non-peptidoglucan components

* The term "murein" as suggested by Weidel and Pelzer [1] is used in this paper synonymously with the expression "peptidoglucan" according to Strominger and Ghuysen [2] to designate the characteristic cell wall polysaccharide consisting of β-1,4-linked *N*-acetyl-muramic acid and *N*-acetyl-glucosamine residues interlinked by peptide bridges.

such as lipoprotein, lipopolysacharide, and teichoic acids. For example, the *Clostridia*, *Corynebacteria*, *Mycobacteria*, and *Propionibacteria* have been reported as requiring a special pretreatment before they can be attacked by lysozyme.[3] In certain instances an increase in the sensitivity to lysozyme was achieved by gentle pretreatment of the cells, *e.g.* by moderate heat, lyophilization, or slight denaturation. In other cases more drastic procedures such as treatment with hot formamide, hot phenol, or trichloroacetic acid were required.[2] Amongst the lactic acid bacteria *Streptococcus faecalis* has been reported to be attacked by lysozyme without any pretreatment,[4-6] whereas *Lactobacillus fermenti* is not susceptible to the direct action of lysozyme (*cf.* Ref. 10). However, cell walls from certain other lactic acid bacteria have been prepared and shown to contain murein as the main component.

Our interest in a selective removal of the cell walls and in obtaining cell membranes from *Lactobacillus fermenti* arose from studies of the active transport of thiamine in this organism.[7-9] Preparation of a particulate material containing fragments of both the cell wall and the cell membrane and exhibiting ATPase activity has been reported earlier from this laboratory.[10] The objective of the present study was to find a suitable pretreatment of *L. fermenti* which would render it sensitive to the action of lysozyme and enable subsequent isolation of the cell membrane. The effects of temperature, pH, butanol, EDTA, urea, and certain proteolytic enzymes as well as that of the age of cells were studied. In those cases where lysis was achieved, fragments presumably corresponding to the cell membrane were isolated by centrifugation at 38 000 *g* and investigated for ATPase activity.

EXPERIMENTAL PROCEDURE

Reagents. Egg white lysozyme, trypsin, adenosine-5′-triphosphate disodium salt (ATP), adenosine-5′-diphosphate sodium salt (ADP), adenosine-5′-monophosphate sodium salt (AMP), and deoxyribonuclease (DNAase) were preparations from Sigma, St. Louis, Mo., USA. Sucrose and sodium pyrophosphate, reagent grade, were purchased from Merck (Darmstadt, Germany). *Proctase* (Japan) was obtained from Kjellbergs Successors AB (Stockholm, Sweden); *Ficoll* from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade. Redistilled water was used throughout the investigation.

Organism and growth media. The maintenance, storage, and cultivation of *L. fermenti* 36 (ATCC 9833) were carried out as previously described.[8] The content of thiamine in the culture medium was 2 mg/l. Unless otherwise stated, the cells were grown for 20–24 h before harvest. Two-step cultivation was usually employed.

Preparation of washed cell suspensions. The cells were harvested by centrifugation, washed once with 0.9 % NaCl, twice with redistilled H_2O and re-suspended in 0.1 M Tris-HCl buffer (pH 7.5), unless otherwise stated. The initial density of the cell suspensions was usually adjusted to 2–30 % transmittance in a Coleman Spectrophotometer, Model 14. More concentrated cell suspensions were investigated on certain occasions mentioned in the text. In certain experiments the cells were osmotically stabilized by sucrose (0.2–0.4 M) or *Ficoll* (10–20 %).

The extent of lysis was measured by turbidimetric determinations followed, in certain experiments, by microscopic examination. Appropriate control tubes with the corresponding untreated cell suspensions were included in every experiment. All determinations were carried out in duplicate, or triplicate, and most experiments were repeated at least once.

Lysozyme was usually added to give a final concentration of 200—400 μg/ml. Considerably lower (10—20 μg/ml) and considerably higher (1—2 mg/ml) concentrations were also investigated, but did not induce lysis. The lysozyme was added directly or after one or several of the pretreatments described below. The time of incubation with lysozyme varied between 2 and 24 h — occasionally still longer as indicated.

Pretreatment by heat. Test tubes, 18 × 200 mm, containing 10 ml of the cell suspension in a suitable buffer with or without the osmotic stabilizer, were heated to 60°C during 15 min and cooled immediately to either 25°C, 37°C, or 45°C before the addition of lysozyme and subsequent incubation.

Pretreatment by butanol, EDTA, or urea. Cells suspended in various buffers were treated with 5 % v/v butanol or *sec.* butanol at 45°C, 50°C, 55°C, or 60°C during 15, 30, 60, or 120 min before the addition of lysozyme. The treatment with EDTA (disodium salt of ethylene diamine tetra-acetic acid) involved heating to 57°C during 30 min or 60 min in the presence of 50, 100, 150, or 200 μg/ml of the agent.

Urea was employed in a 4 M concentration and incubated with the cells at 45°C. In some experiments the cells were centrifuged after the urea treatment, washed once with phosphate buffer and resuspended in the same buffer before subsequent incubation with lysozyme.

Pretreatment by trypsin and certain other proteolytic enzymes. Trypsin was usually employed in a concentration 200 μg/ml. Considerably lower or higher concentrations were used in certain experiments.

Proctase, a preparation from *Aspergillus niger* manufactured in Japan, is a mixture of proteolytic enzymes. It was generously donated by AB Kjellbergs Successors, Stockholm, and employed in certain experiments instead of trypsin, at similar concentrations.

Preparation of membrane-like fragments. The cell suspensions were incubated with trypsin and lysozyme at 45°C without or with an osmotic stabilizer. When the transmittance of the suspensions had increased from about 5 % to about 60—90 %, thus indicating lysis, the suspensions were centrifuged at 38 000 *g* for 15 min and washed with a buffer consisting of Tris (0.1 M)+$MgCl_2$ (0.01 M), pH 7.5, until the protein content of the washings was less than 20 μg/ml. Usually 2—5 successive washings were required. The sediment was resuspended in a few ml buffer (0.2 M Tris, pH 7.5) and stored at +4°C until analyzed for protein content and ATPase activity. Protein was determined according to Lowry *et al.*[11]

ATPase assays. Unless otherwise stated, the reaction mixture contained Tris-buffer 0.1 M (pH 7.5), ATP disodium salt 5×10^{-3} M, $MgCl_2$ 2.5×10^{-2} M, membrane protein 30—250 μg/ml, total volume 2 ml. The reaction mixture was prepared in centrifuge tubes cooled in ice. Incubation was carried out at 37°C, usually during 20 min. The reaction was stopped with 1 ml ice-cold 1 M perchloric acid and the tubes were immediately transferred to the ice bath. The protein precipitate was centrifuged off and inorganic phosphate was determined in the supernatant according to the method of Berenblum and Chain as modified by Martin and Doty.[12] The obtained values were corrected for the spontaneous decomposition of the substrate and for the endogenous phosphate content of the membrane preparations. All ATPase determinations were carried out in duplicate and each experiment was repeated many times.

RESULTS AND DISCUSSION

Lysis of the cells. The course of lysis of washed *L. fermenti* cells under different conditions is shown in Fig. 1. It can be seen that lysis occurs only when both trypsin (200 μg/ml) and lysozyme (200 μg/ml) are present (Curves a or b). Lysozyme alone (Curve d) has no lytic effect, causing rather an increase in the turbidity. The effect of trypsin alone is negligible (Curve c). Control suspensions without added enzymes, whether with or without pretreatment by heat, did not undergo lysis (Curve e; *cf.* also the control suspensions to the different experiments illustrated in Figs. 2—7).

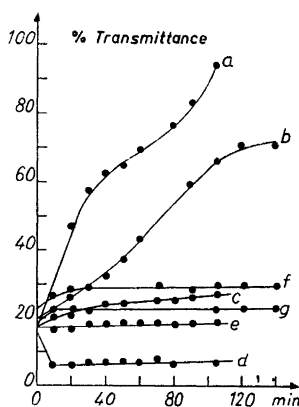

Fig. 1. The influence of the age of cells and the addition of trypsin (T) and lysozyme (L) to washed *L. fermenti* cells suspended in Tris-HCl, 0.1 M (pH 7.5). Cells pretreated by heat at 60°C for 15 min. a—e: incubation at 45°C; f—g: incubation at 48°C.

a. L 200 μg/ml, T 200 μg/ml, cells grown for 24 h;
b. as a, but cells grown for 48 h;
c. as a, but L omitted;
d. as a, but T omitted;
e. as a, but both L and T omitted;
f. as a, but L 10 μg/ml, T 20 μg/ml;
g. as f, but no heat pretreatment.

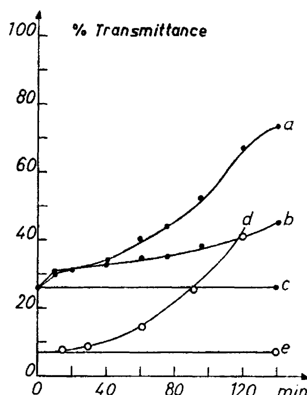

Fig. 2. The influence of magnesium chloride, and sulphate on the course of lysis of washed *L. fermenti* cells in the presence of trypsin (T) and lysozyme (L). Mg-salt 0.01 M. Other conditions as in Fig. 1.
a. Tris-HCl; b. as a, but with $MgSO_4$; c. control suspensions as a or b, respectively, but T and L omitted; d. as a, but with $MgCl_2$; e. as d, but T and L omitted.

Furthermore, it can be seen in Fig. 1 that lower levels of the enzymes (20 μg/ml of trypsin and 10 μg/ml of lysozyme) are without effect (Curve f). Other experiments indicated that increasing the lysozyme level from 200 to 400 μg/ml as employed by several authors (*cf.* Ref. 3) did not substantially increase the efficiency of lysis. At enzyme levels of 2000 μg/ml inhibition of lysis could be observed. In all subsequent experiments reported here levels of 200—400 μg lysozyme/ml and 200 μg trypsin/ml were used.

Fig. 1 also shows the effect of the age of cells on the course of lysis in the presence of lysozyme and trypsin. It is seen that cells harvested after 24 h of growth (Curve a), corresponding to the final stages of the logarithmic phase, lyse more rapidly than cells grown for 48 h (Curve b), *i.e.* taken from the stationary phase. In most subsequent experiments reported here, cells grown for 18—24 h were employed.

The effects of magnesium sulphate and chloride are illustrated in Fig. 2. It is seen that sulphate depresses the extent of lysis as compared to the effect of chloride, whereas magnesium seems to be without effect on the lysis. However, magnesium chloride (0.01 M) was used as a standard buffer component in all subsequent experiments reported here, because the integrity of the cell membrane requires this ion as is also the case with the ATPase reaction.

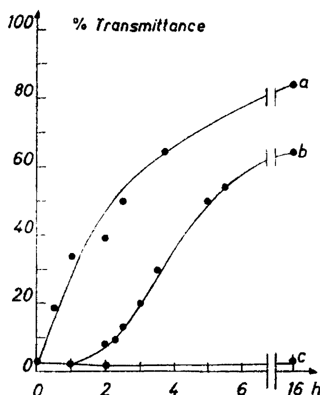

Fig. 3. The influence of an osmotic stabilizer on the course of lysis of washed *L. fermenti* cells in the presence of trypsin (T, 200 μg/ml) and lysozyme (L, 400 μg/ml). Tris-HCl 0.1 M pH 7.5 containing $MgCl_2$ 0.01 M, incubation at 45°C.
a. no further additions; b. sucrose 0.4 M; c, as a, but either T or L omitted.

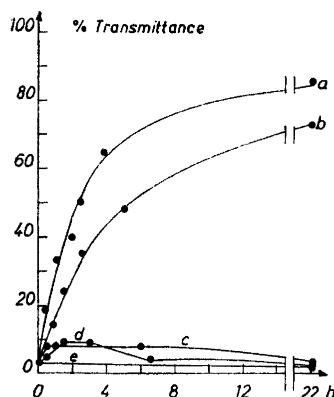

Fig. 4. The influence of temperature on the course of lysis of washed *L. fermenti* cells. Conditions as in Fig. 3a.
a. incubation at 45°C; b. incubation at 37°C; c. as a, but T omitted; d. as b, but T omitted; e. as either a or b, but either L or both T and L omitted.

The influence of an osmotic stabilizer on the course of lysis is illustrated in Fig. 3. It is seen that the lysis is less efficient in the presence of 0.4 M sucrose than it is in its absence, other conditions being identical. *Ficoll* (10 %) had essentially the same effect as sucrose.

The effect of temperature in the absence of an osmotic stabilizer is shown in Fig. 4. It is seen that lysis at 37°C was slower than at 45°C. In the presence of 0.4 M sucrose the difference was even more pronounced. Since the extent of lysis decreased with temperature and in the presence of sucrose, lysis at 45°C and in the absence of an osmotic stabilizer was selected as a standard condition in most subsequent experiments reported here, whereas osmotically stabilized media were used only occasionally. In such experiments the occurrence of spherical protoplasts could be observed alongside with a minor proportion of irregularly shaped bodies. In control suspensions, incubated with trypsin alone or without any enzyme addition, only rod shaped organisms could be detected.

The influence of pH on the course of lysis is shown in Fig. 5, which depicts a typical experiment. In this case the lysis was carried out in the presence of sucrose. A similar dependence on the pH was observed in media that were not osmotically stabilized. It can be seen in Fig. 5 that for the pH values investigated the lysis was most rapid at pH 8.0, somewhat less so at pH 7.5 and still slower at pH 6.9. With regard to the objective of isolating intact cell membranes, pH 7.5, was selected for the standard procedure. However, establishment of a theoretical optimum pH value for the lysis is a complicated matter, because optimal conditions for the action of both trypsin and lysozyme have to be satisfied.

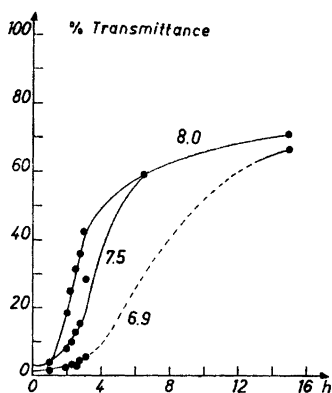

Fig. 5. The influence of pH on the course of lysis of washed *L. fermenti* cells suspended in Tris-HCl 0.1 M containing $MgCl_2$ 0.01 M, sucrose 0.4 M, trypsin 200 μg/ml, and lysozyme 400 μg/ml. Incubation at 45°C. The figures denote pH values.

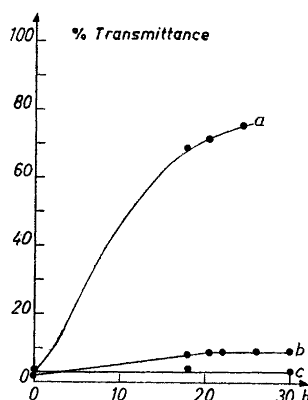

Fig. 6. The influence of pretreatment with EDTA (100 μg/ml) or trypsin (T, 200 μg/ml) on the susceptibility of washed *L. fermenti* cells to lysozyme (L, 400 μg/ml). Incubation at 45°C in Tris-HCl, 0.1 M (pH 7.5) containing $MgCl_2$, 0.01 M.
a. EDTA+T+L; b. EDTA+L; c. EDTA.

The pretreatment with butanol (5 % v/v) or urea (4 M) as described under *Experimental Procedure* did not give any substantial sensitivization towards lysozyme. With EDTA a slight increase in sensitivity to lysozyme could be observed. The effect of EDTA is illustrated in Fig. 6; that of urea in Fig. 7.

In the presence of *Proctase* the cells became sensitive to lysozyme. However, when used at the same level as trypsin (200 μg/ml) *Proctase* was approximately only half as efficient as trypsin. This is illustrated in Fig. 8 when the lysis was carried out at pH 6.9 instead of the usual pH 7.5. The observed difference may be only apparent, due to the different optimum conditions required for the two enzyme preparations. *Proctase* is a rather indefinite mixture of proteolytic enzymes. According to the manufacturer the optimum pH for this preparation is around 3.5.

In conclusion, it can be said that, of the different conditions investigated, only proteolytic action renders washed *L. fermenti* cells essentially susceptible to lysozyme. The shortest time required to lyse a suspension from about 5 % to about 80 % transmittance (Coleman reading) was about 2—5 h at 45°C in the presence of 200 μg trypsin and 400 μg lysozyme per ml. In many cases, however, particularly with more aged cells, at lower temperature etc., the corresponding time was considerably longer (*cf.* Figs. 2—7).

It thus appears that the cell wall of *L. fermenti* differs considerably from such "classical" lysozyme substrates as the walls of *Micrococcus lysodeikticus* or *Bacillus megaterium* which are easily lysed by lysozyme within 1—2 h (*cf.* Ref. 3).

Preliminary experiments were carried out to study the material which is removed from the cell surface of *L. fermenti* by trypsin before the attack of lysozyme can take place. Supernatants from cell suspensions incubated with

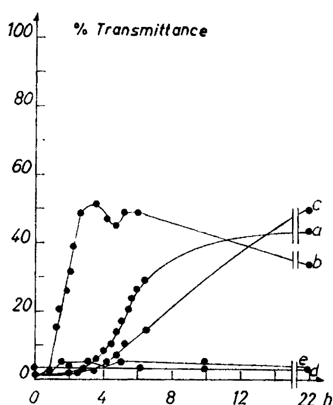

Fig. 7. The influence of pretreatment with urea (U, 4 M) or trypsin (T, 200 μg/ml) on the susceptibility of washed *L. fermenti* cells to lysozyme (L, 400 μg/ml). Incubation at 45°C in potassium phosphate (pH 7.5) containing $MgCl_2$, 0.01 M, at the following molar concentrations of phosphate.
a. 0.1 M, T+L; b. 0.04 M, T+L; c. 0.04 M+ sucrose 0.4 M, T+L; d. 0.04 M, U or U+L; e. 0.04 M, L only.

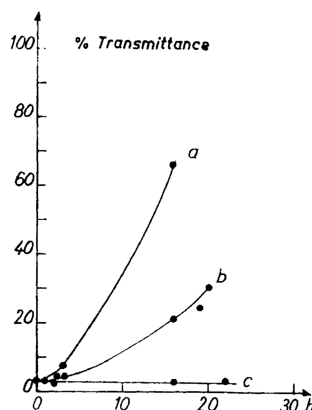

Fig. 8. The influence of *Proctase* (P, 200 μg/ml) and trypsin (T, 200 μg/ml) on the susceptibility of washed *L. fermenti* cells to lysozyme (L, 400 μg/ml). Incubation at 45°C in Tris-HCl, 0.1 M (pH 6.9), containing $MgCl_2$ 0.01 M and sucrose 0.4 M.
a. T+L; b. P+L; c. L.

trypsin, cell suspensions alone and control solutions of trypsin, incubated under identical conditions, were lyophilized before or after precipitation of proteins with trichloroacetic acid (TCA). The concentrated material was analyzed for amino acids by two-dimensional paper chromatography after or without preceding hydrolysis. The supernatants from cell suspensions alone gave spots corresponding to alanine, aspartic acid, and two unidentified substances. The supernatants from trypsin solutions alone or trypsin plus cell suspensions, after precipitation with TCA, both gave spots corresponding to phenylalanine, tyrosine, leucine, isoleucine, valine, alanine, aspartic acid, and three unidentified substances. Further work is in progress to elucidate this problem.

The ATPase activity of isolated membrane-like fragments. The liberation of inorganic phosphate (P_i) at varying time intervals of incubation of the membrane-like preparations with ATP is shown in Fig. 9. It can be seen that the reaction velocity increases linearly with time during 1—2 h. In most experiments reported here an incubation time of 20 min was selected as representing the initial reaction velocity. ATP-hydrolyzing activity was proportional to the amount of membrane protein up to 70—100 μg/ml (Fig. 10).

The results of determinations of P_i-releasing activities of a number of membrane-like preparations are shown in Table 1. It can be seen there that all but one of the preparations exhibited a distinct ATP-hydrolyzing activity, which was rather specific with respect to ATP. No activity was obtained with

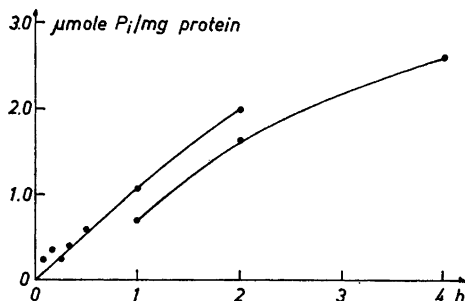

Fig. 9. The P_i releasing activities of a membrane-like preparation from *L. fermenti* at varying time intervals. Incubation mixture: Tris-HCl, 0.1 M (pH 7.5); ATP 5×10^{-3} M; $MgCl_2$ 2.5×10^{-2} M; membrane protein 125 μg/ml, total volume 2 ml. Incubation at 37°C. The two curves represent incubations carried out on two different days using two different preparations.

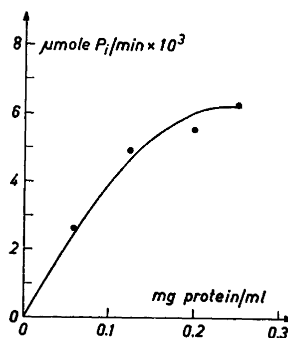

Fig. 10. The relationship between the content of membrane protein in the incubation mixture and the amount of liberated P_i. Incubation for 20 min as in Fig. 9 except for the protein content.

ADP, AMP, or inorganic pyrophosphate as substrates. The specific ATPase activities varied usually between 0.02—0.09 μmole P_i/mg protein/min with extreme values of 0.01 and 0.22, respectively. The conditions of cell lysis

Table 1. The P_i-releasing activities of membrane-like preparations from osmotically lysed cells of *L. fermenti* using various substrates (5×10^{-3} M). Enzyme assay as in Fig. 9 using 50—125 μg protein/ml.

Preparation No.	Hours of incubation of the cells with trypsin alone	Hours of incubation of the cells with trypsin plus lysozyme	P_i liberated by the corresponding membrane preparations in the presence of ATP	ADP	AMP	PP
			μmoles/mg protein/min			
1	3	57	0.05			
2	18	42	0.22			
3	0	20	0.09			
4	0	26	0.03			
5	20	24	0.03	0.00	0.00	0.01
6	20	24	0.00	0.00	0.00	0.00
7	18	48	0.02	0.00	0.00	0.00
8	18	24	0.01	0.00	0.00	0.00
9	18	24	0.03	0.00	0.00	0.00
10	19	25	0.02			
11	18	24	0.02			
12	0	5.5	0.02			
13	0	4.75	0.03			
14	0	5.0	0.04			
15	0	3.75	0.05			

before preparation of the membrane-like fragments were varied in certain experiments, but no clear-cut correlation between these variations and the ATPase activities of the final preparations could be observed. Lower ATPase activities were obtained after extended time of the cell lysis with some preparations, but not with others. Considerable variations in ATPase activities between particulate materials prepared in an identical way from different batches of *L. fermenti* were observed earlier as well.[10]

Whereas the previously reported type of preparations, obtained from mechanically disrupted cells, exhibited, in addition to the ATP-hydrolyzing activity, also inorganic pyrophosphatase activity,[10] this is not the case with the type of preparation described here and obtained by lysis with lysozyme and trypsin. Another difference between the two types of preparation is the over-all lower specific ATPase activity exhibited by membrane-like fragments prepared by controlled lysis (*cf.* Table 1 and Ref. 10). It cannot be decided at present whether these differences represent some intrinsic properties of the *L. fermenti* membranes or whether they are a result of the relatively long time at 45°C required in the present procedure for a selective removal of the cell wall. Further, the proteolytic treatment required in order to render the cells sensitive to lysozyme may also have a partially detrimental effect on the membrane proteins including the ATPase and pyrophosphatase.

Determinations of the ATPase activities of the supernatants and washings after the separation of the membrane-like fragments indicated that substantial amounts of ATPase were present in the soluble form, either originally or possibly after release from the mebranes during fractionation procedures.

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