Studies on Sucrose-Induced Autolysis of Clostridial Cells : Part 3. Autolytic Formation of Clostridial Protoplasts (Autoplasts) as Revealed by Microscopy and Their Some Properties

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Studies on Sucrose-Induced Autolysis of Clostridial Cells

Part 3. Autolytic Formation of Clostridial Protoplasts (Autoplasts) as Revealed by Microscopy and Their Some Properties

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When exponentially growing cells of ***Clostridium saccharoperbutylacetonicum*** (ATCC 13564) were exposed to hypertonic concentration of sucrose (0.3-o. 5 M), rapid degradation of the cell wall occurred (sucrose-induced autolysis). The morphological changes from the original rod-shaped cells to protoplast-like cells during the sucrose-induced autolysis were observed by phase contrast and electron microscopy. When the cells were autolysed in the sucrose solution (0.35 M), each cell began to swell at the middle or at one pole and then formed a small bulb at the swollen part. The bulb gradually enlarged as lysis progressed, and finally became a protoplast-like cell (PLC) which had no cell wall. The larger pre-division cell formed the bulb at the middle (septal site), while the small post-division cell formed the bulb at the pole. PLCs had the following properties: (1) they were osmotically fragile and liberated cytoplasmic components into the suspending fluid by osmotic shock; (2) they were effectively stabilized by 0.4 M sucrose solution containing 5 mM Mg^{2+}; (3) they could not adsorb phage particles, but they were infected by phenol-extracted phage DNA; (4) they, as same as parent (normal) cells, produced an inducible bacteriocin (clostocin 0) after treatment with mitomycin C; (5) they were able to grow volumentrically and became larger by continuous cultivation in stabilizing liquid medium, but were unable to multiply; and (6) they synthesized all their macromolecules (DNA, RNA, protein and lipid), but DNA synthetic ability became weak as cultivation progressed. From these results, they were regarded as living protoplasts (autoplasts).

INTRODUCTION

This work started from an observation of unexpected premature lysis of early-exponentially growing cells of ***Clostridium saccharoperbutylacetonicum*** (ATCC 13564) and ***Clostridium*** *sporogenes* (IFO 12636) in the usual penicillin lytic system used for the formation of bacterial protoplasts. It was considered that the premature lysis was due to the hypertonic concentration of sucrose (0.3-o. 5 M, maximum rate at 0.35 M) added as osmotic stabilizer for the developing protoplasts expected (Ogata ***et al.,*** 1975). We named this specific lysis sucrose-

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induced autolysis. During the sucrose-induced autolysis, the clostridial cells were converted into protoplast-like cells (PLCs).

There have been many reports on the method for formation of bacterial protoplasts. A technique which degrades the rigid cell wall peptidoglycan layer, directly or indirectly, can be employed in protoplast formation. For direct degradation of the cell wall layer, murolytic enzymes such as egg white lysozyme (Weibull, 1953, 1963), phage lysin (Calandra *et al.*, 1975), serum enzyme (Amano, 1968), sporulation and germination enzyme (Schindler, 1965) and other microbial lytic enzyme (Kusaka, 1975) were used. For indirect degradation of cell wall layer, antibiotics such as penicillin (Lederberg, 1956; Kaback and Stadtman, 1966) and D-cycloserine (Bourgesis and Beaman, 1976) which interiere biosynthesis of the peptidoglycan were frequently used. Sometimes, protoplasts were formed by autolysis occurring without any supplement of substances to degrade the rigid cell wall (Joseph and Schockman, 1974, 1976). In this method, degradation of cell wall depend upon the lytic action of autolysin.

There are a few bacterial forms (such as spheroplasts and L-forms; Amano, 1968; Kingam, 1968) resemble to the protoplasts, microscopically and osmotically. So, it is desirable to clarify the properties of developed spherical cells especially when they are formed by new technique. Our techenique for preparation of PLCs is unique in using sucrose as the inducer of rapid bacterial autolysis, and simultaneously as a hypertonic solute for developed PLCs. Therefore, we investigated the detailed process of cellular conversion into PLCs and especially the site of the beginning of cellular lysis, in order to learn the role of sucrose and lytic enzyme (autolysin) in the specific phenomenon. Furthermore, we should examine properties of PLCs to know whether or not PLCs are bacterial protoplasts.

Bacterial protoplasts are absent in the rigid cell wall and osmotically fragile (Weibull, 1963; Martin, 1963). Usually they succeed many of the biological properties of parent cells. However, as they have no cell wall, they also loss some biological properties, for example, the lack of ability for the adsorption of phage particles, of which receptors locate on cell wall. Also they can hardly rebuild the rigid cell wall and multiply (Weibull, 1963; Conser and Marquis, 1969). Usually they have spherical shape, although their parent cells are not spherical (Weibull, 1963; Martin, 1963). For the specific properties of bacterial protoplasts, they have been widely used in the researches on the structure and function of bacterial surface (Ghuysen, 1976; Eda *et* al., 1976), protoplast fusion and bacterial heredity (Sakaguchi, 1979; Ochi, 1979), pathogeny and immune response in some pathogenic bacteria (Rosental *et al.*, 1975), and some other plasmic body-related properties (Mitra *et al.*, 1975; Speicer and Spooneri, 1974). Furthermore, they have frequently used for preparation of bacterial protoplasmic membrane (Schuldiner *et al.*, 1975; Yamamoto *et al.*, 1975). It is said that the protoplasts produced autolytically are better for the preparation of protoplasmic membrane by eliminating the contamination of non-cellular supplements used for degradation of cell wall. From the advantages of autolytically produced protoplasts, Joseph and Shockman (1974) called

them "autoplasts" which distinguished the autolytically produced protoplasts from those produced by using lytic enzymes or antibiotics.

MATERIALS AND METHODS

Bacterial strains

Clostridium saccharoperbutylacetonicum N1-4 (ATCC 13564) was used without otherwise mention. C. *saccharoperbutylacetonicum* N1-504 (ATCC 27022) and N1-611 were used for transfection experiment. These strains are resistant mutants of strain Nl-4 against the adsorption of phage HM 2 (Hongo and Murata, 1965). C. *saccharoperbutylacetonicum No. 8* was also used as a sensitive strain for a bacteriocin clostocin 0 (Ogata *et al.*, 1972, 1976).

Media and cultural conditions

Bacterial cells were grown at 30°C under a reduced atmospheric condition (5-10 mmHg) in TYA medium as described in previous paper (Ogata ***et al.***, 1980). For the preparation of exponentially growing cells, initial optical density (OD_{660}) at 660 nm of culture was adjusted to 0.1 in a fresh TYA medium and incubation was continued until OD_{660} became 0.3. OD_{660} of the culture was measured with a photoelectric colorimeter (model 7A, Tokyo Koden Ltd.). A bilayered solid TYA medium was also used in the experiments for colony formation, and assay of the activity of phage and bacteriocin. The medium contained 1.5 % agar for lower layer and 0.6 % for upper layer.

Induction of cellular autolysis and preparation of protoplast-like cell (PLC)

The cells harvested by centrifugation (10, 000 × *g* for 10 min at room temperature) were gently resuspended in fresh TYA medium or 1/15 M (or 1/30 M) phosphate buffer (pH 6.5, containing 5 mM Mg^{2+}) containing 0.4 M sucrose, and then incubated at 30°C. Lysis was followed by measuring the OD_{660} of the culture as described above. More than 99% of the cells were converted into PLCs after 2 hr of autolysis. Produced PLCs were harvested by centrifugation (3,000 xg for 20 min) at room temperature and then resuspended in TYA medium or buffer solution containing 0.4 M sucrose with or without addition of 5 mM Mg^{2+}.

Phase contrast microscopy

Samples of the culture were withdrawn at 5-min intervals, and the cells were immediately fixed with 5 % (v/v) formalin for 10 min at room temperature. The fixation was carried out to prevent further lysis of the cells. Photographs were taken with a phase contrast microscope (Nippon Kogaku Kogyo Ltd.).

Electron microscopy

The cells were fixed with ice-cold osmic acid dissolved in veronal acetate buffer (pH 6.0). Concentration of the fixative was adjusted to 0.1 and 1.0 % (w/v) for pre- and post-fixation, respectively. The fixed cells were embedded

in 2% agar, and then dehydrated in a series of ethanol concentrations (50, 70, 90, 95 and 100 %). They were embedded again, in the resin Epon 812. The resin was polymerized by gradual raising the temperature (40, 55 and 60°C). The cells were thin-sectioned with an ultramicrotome (Sorvall, MT-2), and then stained with 1.0 % uranyl acetate and lead acetate. Photographs were taken with an electron microscope (JEM 100B, Japan Electron Optics Laboratory Ltd.).

Procedure of osmotic shock

PLCs harvested from 100 ml of autolysate were rapidly suspended in the same volume of deionized water. The sensitivity of PLCs to osmotic shock was investigated by measuring the turbidity of the cell suspension before and after shock. After centrifugation (10, $000 \times g$ for 10 min) of cell suspension, supernatant fluid was measured at 260 and 280 nm.

Adsorption analysis of phage particles on protoplast-like cell (PLC)

Phages HM 2 and HM 3 (Hongo and Murata, 1965), active on strain Nl-4, were added to PLC culture with various multiplicity of infection (m.o.i.), and then the culture was incubated at 30°C. After 15 min of incubation, the culture was immediately centrifuged (10, $000 \times g$ for 10 min) at **2°C.** Phage particles remaining in the supernatant fluid were assayed according to the double layer plate technique.

Colony formation

PLCs were plated on the solid TYA medium after dilution with buffered sucrose solution, and then incubated for 15 hr at 30°C with reduction of the atmospheric pressure.

Fractionation and analysis of macromolecular content

Macromolecules of PLCs were fractionated according to the modified STS (Schmidt-Trannhauser-Schneider's) method (Mizuno, 1974) after precipitation of PLCs with 10 % (w/v) cold TCA (trichloroacetic acid). The content of DNA, RNA and protein was analysed according to Burton's diphenylamine method (Mizuno, 1974), Mejbaum's orcinol method (Mizuno, 1974) and Lowry's phenol-reagent method (Sugawara and Soejima, 1977), respectively.

Analysis of incorporation of radio-labelled precursor into macromolecules

PLCs were cultivated in the presence of 2.5×10^{-3} mCi/ml of ^{32}P-orthophosphoric acid or 1. Ox 10^{-3} mCi/ml of methyl-^{3}H-thymidine. An aliquot of the culture was withdrawn at intervals and then PLCs were precipitated by using ice-cold TCA (5%). After washing once with the same acid solution, the precipitation containing ^{3}H was decomposed for 15 hr with N KOH at 37°C. Radioactivity was measured with a scintillation counter (model LS-250, Beckman). The other precipitation containing ^{32}P was fractionated into DNA, RNA and lipid fraction, and radio-activity was measured with a gas flow counter (model TDC-10, Aloka). Radioisotopes were purchased from Daiichi Radioiso-

tope Lab. Ltd.

Induction of bacteriocin clostocin 0

PLCs were cultivated for 3 hr in the presence of mitomycin C (0.1 to 1.0 μg/ml, Kyowa Hakko Kogyo Co.) and then centrifuged (3, 000$\times g$ for 10 min) to remove the PLCs. Supernatant fluid was spotted on solid TYA medium on which indicator bacteria (strain No. 8) were previously inoculated. The activity of an inducible bacteriocin clostocin 0, produced by C. *saccharoperbutylacetonicum* Nl-4 (Ogata *et al., 1972,* 1976), was represented as an arbitrary unit of activity (units/ml) which was defined as the reciprocal of the highest dilution clearly showing inhibition zone by spot test. One unit contains approximately 1×10^8 particles/ml of clostocin 0.

Preparation of infectious bacteriophage DNA

Phage DNA was isolated from purified phage HM 2 preparation according to phenol method. The details on the preparation of DNA is described in Fig. 1. The preparation contained 2 to 3 mg/ml of DNA. A decomposed DNA was also prepared by DNase treatment (5 μg/ml, type I, Sigma).

Transfection

An aliquot of DNA preparation (0.2 ml) was added to PLCs culture (2ml)

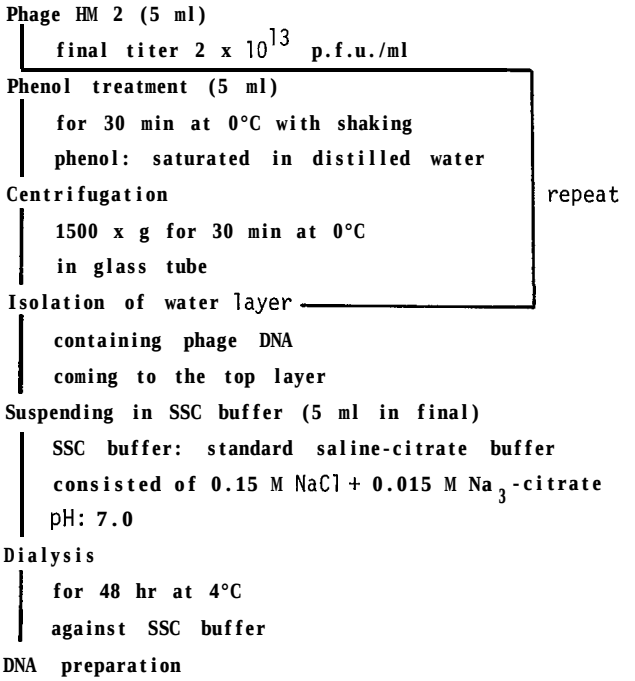

Fig. 1. Procedure of phage DNA isolation. The final DNA preparation contained 2 to 3mg of DNA/ml. It did not have any infectious phage particles.

which contained about 2 x 10^8 cells/ml. The infected PLCs were cultivated for 3 hr with reduction of atmospheric pressure. After the cultivation, PLCs were removed by centrifugation (10, 000 × *g* for 10 min at 2°C) and the supernatant was plated with the indicator bacteria (strain Nl-4) to assay the liberated phage particles.

RESULTS

Observation with phase contrast microscope

The morphological changes in the cells were followed by phase contrast microscopy. The morphological changes were characterized by the time course of lysis, as shown in Fig. 2. The cells (Fig. 2 A) were original rod shape (3-6 x 0.4-O. 6 μm). During the first 10 min of incubation with sucrose in TYA medium (Fig. 2 B), the cells began to swell at one pole or the middle, and cell formed a bulb at the swollen part. The cells that swelled initially at the middle were about 1.5–2.0 times larger than the cells that swelled initially at the pole. The ratio between the number of the cells swelling initially at the

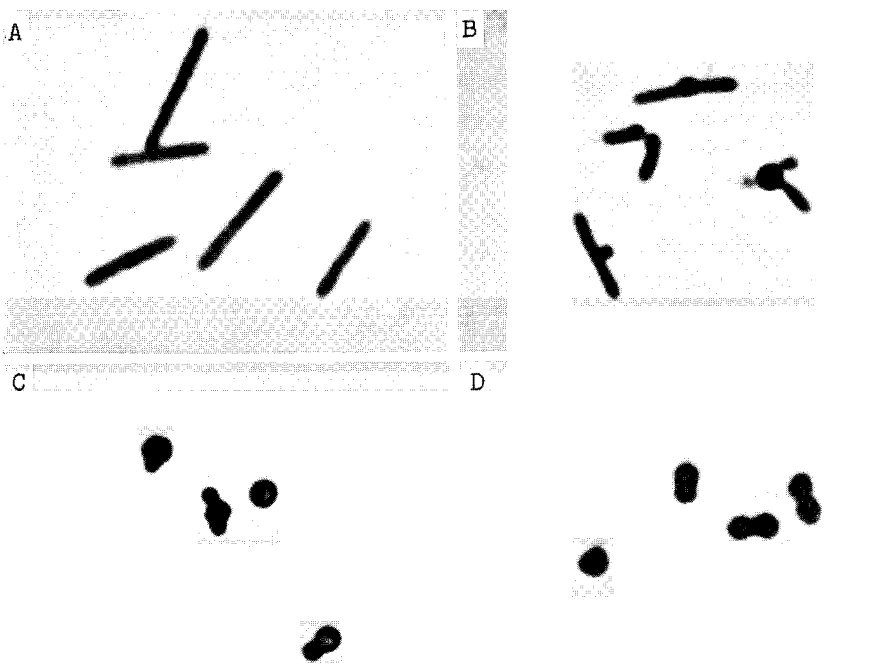

Fig. 2. Morphological changes in *Clostridium saccharoperbutylacetonicum* observed with the phase contrast microscope during sucrose-induced autolysis. Photographs were taken with a phase contrast microscope before (A) and after incubation for 10 min (B), 20 min (C), and *40* min (D) with sucrose. Scale indicates 5 μm.

middle and at the pole was about 7 to 3. We did not observe bulbs on both poles of the cell. This may indicate that the degradation velocity of the cell wall on the two poles is unequal, or that the degradation occurs only on the pole where there was a newly synthesized wall. Between 10 and 20min (Fig. 2 C), the bulb gradually enlarged. On the other hand, the remaining part of the rod became smaller. The cell swelling at the middle frequently had parts of the rod on both sides of the enlarged bulb. Between 20 and 40min (Fig. 2 D), protoplast-like cells (PLCs) 1.0-l. 5 μm in diameter were observed. After 40 min, more than 99 % of the cells were converted into PLCs.

Observation with electron microscope

The morphological changes in the cells were also followed by examining thin sections by electron microscopy.

The cells were fixed with ice-cold after 2min of incubation with sucrose suspended in phosphate buffer, to make clear the sites where the morphological changes began. Each cell extruded its cytoplasmic material at the pole (Fig. 3 A, C and E) or at the middle (Fig. 3 B and D), while the original

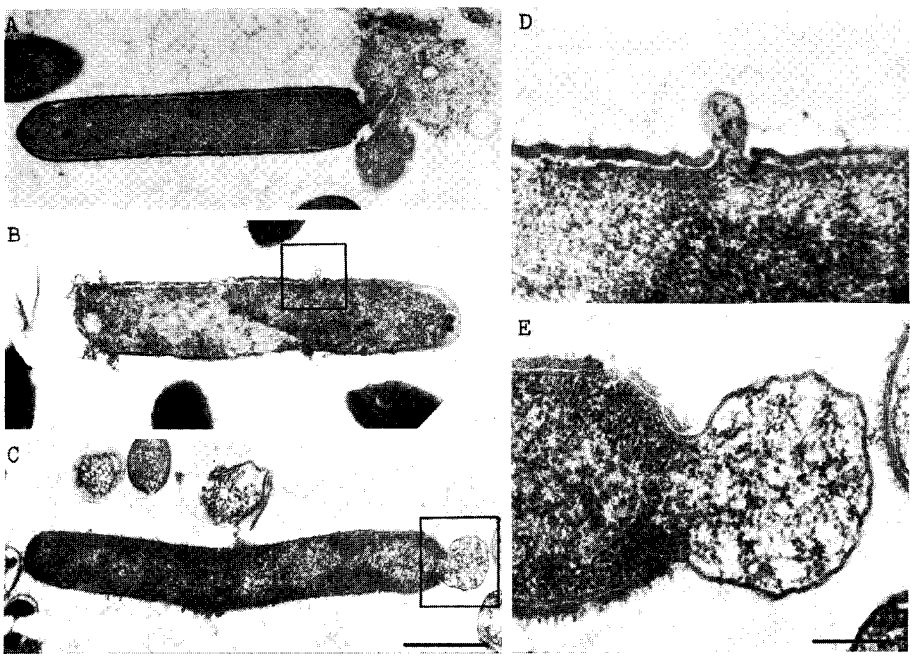

Fig. 3. Beginning site of morphological changes in *Clostridium saccharoperbutylacetonicum* during sucrose-induced autolysis. Harvested cells were fixed with a large volume of ice-cold osmic acid to stop the rapid lysis after 2 min of incubation with sucrose. Thus, *sucrose* concentration was immediately lowered to 0.2 M or less, and sometimes the cytoplasmic membrane was broken. Photographs D and E are enlargements of the outlined areas in photographs B and C, respectively. Scales indicate 1 μm (A, B and C) and 0.2 μm (D and E).

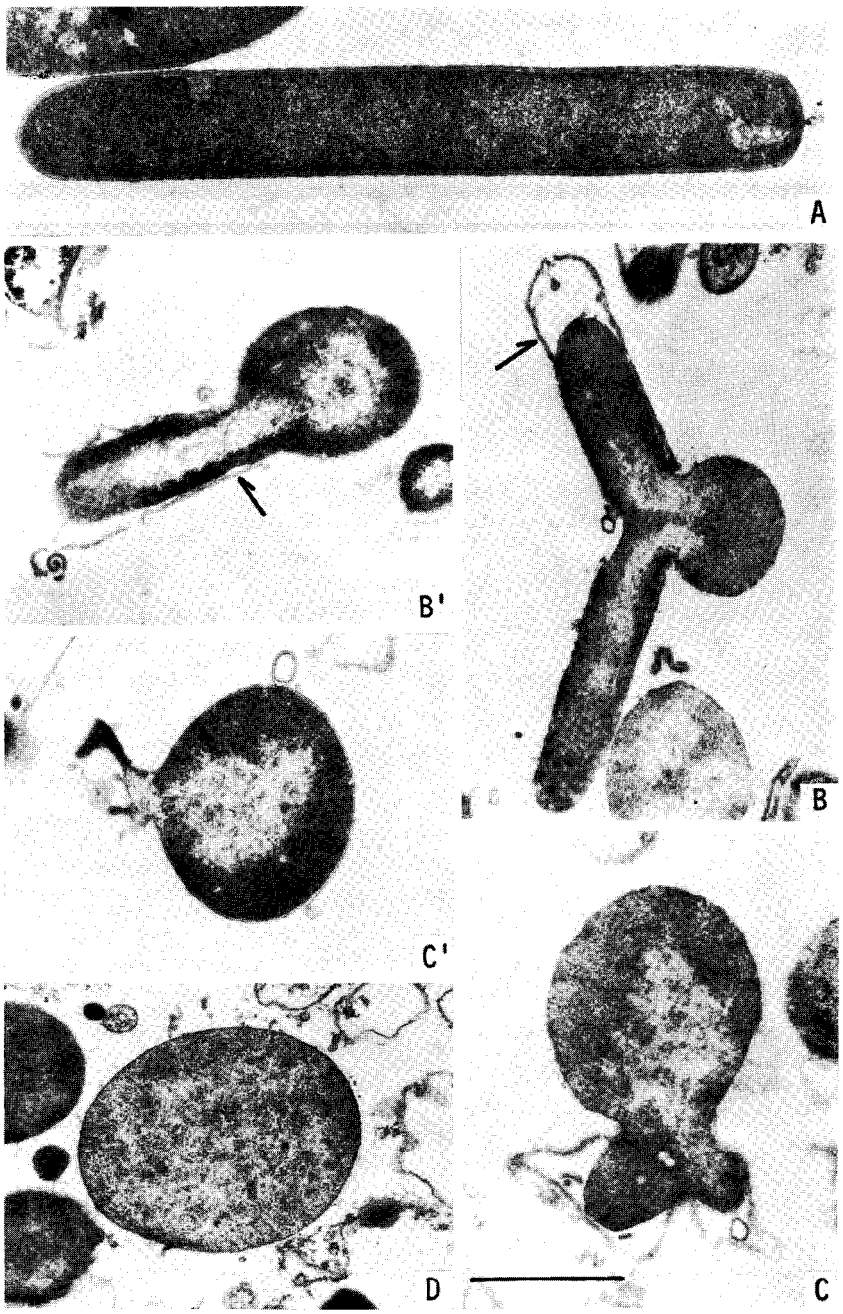

Fig. 4. Morphological changes in *Clostridium saccharoperbutylacetonicum* observed with the electron microscope during sucrose-induced autolysis. Photographs were taken with an electron microscope after thin-sectioning the cell. Cells were fixed before (A) and after incubation for 10 min (B and B'), 20min (C and C'), and 40min (D) with sucrose. Scale indicates 0.5 μm. The cells were converted into protoplast-like cells along the lines of A→B→C→D or A→B′→C′→D. Arrows show cell wall.

cell (Fig. 4 A) was entirely surrounded with the smooth cell wall layer which was tightly attached to the plasma membrane. It was noticed that the site of the extruding cytoplasm was defined at the pole or middle (septal site) where the cell wall was broken. This result indicates at least two basic aspects of the lysis: first, morphological changes in the cell are based on the degradation of the rigid cell wall; second, the degradation of the cell wall occurs predominantly at one pole or at the middle of the cell.

During the first 10 min incubation with sucrose in phosphate buffer (Fig. 4 B and B'), a bulb was observed at the middle or at the pole of cell. The bulb was composed of cytoplasmic components which had been extruded from the cytoplasm and covered with only the cytoplasmic membrane. The cell wall was seen only on the part of the rod which was not yet converted but it did not keep its normal shape. The cell wall was sometimes coiled and broken at several points, while that of the original cell was smooth and not broken (Fig. 4 A). Between 10 and 20 min (Fig. 4 C and C'), most of the cell wall was gradually removed from the cell surface, and the bulb gradually enlarged. Between 20 and 40min (Fig. 4 D), the cell became a large spherical body, the PLC, which had no cell wall. All of these observation corresponded well with those made with the phase contrast microscope.

Physical properties of prtoplast-like cell (PLC)

It is necessary for experiments with PLCs to understand firstly the conditions to stabilize the PLCs. Therefore, osmotic properties of PLCs was initially investigated.

(1) Osmotic sensitivity of PLC

Osmotic shocked PLC culture lost more than 90 % of its initial turbidity (OD_{660}), while OD_{260} and OD_{280} of the supernatant increased to about three times of control, as shown in Table 1. This result indicates that cytoplasm of osmotic shocked PLCs leak out to the supernatant by bursting of their envelop. As shown in Fig. 5, the shocked PLCs show only bilayered protoplasmic membrane, while in the case of the control, cytoplasm enveloped by protoplasmic membrane was visible. These results indicate that PLCs **are** sensitive to osmotic shock.

Table 1. Changes of optical density of protoplast-like cell suspension by osmotic shock. After 5 min of incubation at room temperature, the shocked PLC suspension was measured at 660nm. A portion of the suspension was centrifuged (10, 000 × *g* for 10 min at 2°C) and the supernatant fluid was measured at 260 and 280nm. For control system, a buffered sucrose solution (0.40 M sucrose) was used instead of distilled water. Osmotic shock was carried out as described in the Materials and Methods.

Wave	Shocked system	Control system
660 nm	0.03	0.38
260	2.76	0.88
280	1.44	0.50

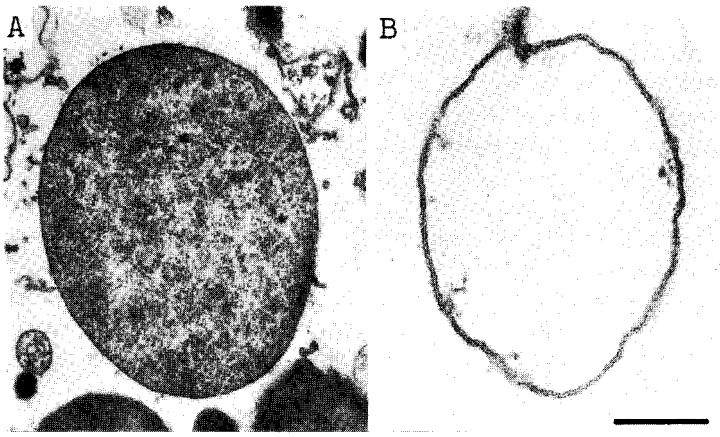

Fig. 5. Morphology of protoplast-like cell before and after osmotic shock. Photo. A shows the morphology before osmotic shock, while photo. B shows that after osmotic shock. Scale line indicates 0.5 μm.

(2) *Effect of sucrose and Mg^{2+} concentrations on the stabilization of PLC*

It is known that protoplasts are easily prevented from their disruption in a suitable hypertonic solution. Increased concentrations of sucrose and Mg^{2+} also have effect on the stabilization on the protoplasts. Experiments were carried out to find out the optimum concentrations of sucrose and Mg^{2+} to stabilize the PLCs. The experimental procedure was illustrated in Fig. 6. In the procedure, OD_{260} value of the supernatant before and after osmotic shock depended upon the number of bursted PLCs and stabilized PLCs, respectively, during 30min incubation before osmotic shock.

As shown in Fig. 7, the supernatant fluid had the lowest absorbance before osmotic shock and the highest absorbance after osmotic shock at 0.40 and 0.45 M sucrose. The sum of OD_{260} value before and after osmotic shock was almost constant at each concentration of sucrose. This result indicates that these concentrations of sucrose solution is the most effective solution for the stabilization of PLCs.

A resemble result was gained in the experiment with Mg^{2+}. As shown in Fig. 8, increased concentration of Mg^{2+} up to 5mM exhibited a good effect on the stabilization of PLCs. A buffer solution containing 0.4 M sucrose and 5 mM Mg^{2+} was settled as the standard solution for handling with PLCs. The same results had been demonstrated on the protoplasts of *Streptococcus faecalis* (Joseph and Shockman, 1974) and other bacteria (Weibull, 1963; Martin, 1963).

(3) *Resistance of PLC against phage adsorption*

Phage HM 2 and HM 3 used are virulent phages having their receptors on the cell wall of strains Nl-4. Phage adsorption experiment was carried out using these two kinds of phages to understand whether or not the PLCs have cell wall on their surface. As shown in Table 2, PLCs had only a poor adsorption of phage particles. Normal cells were adsorbed almost phages added.

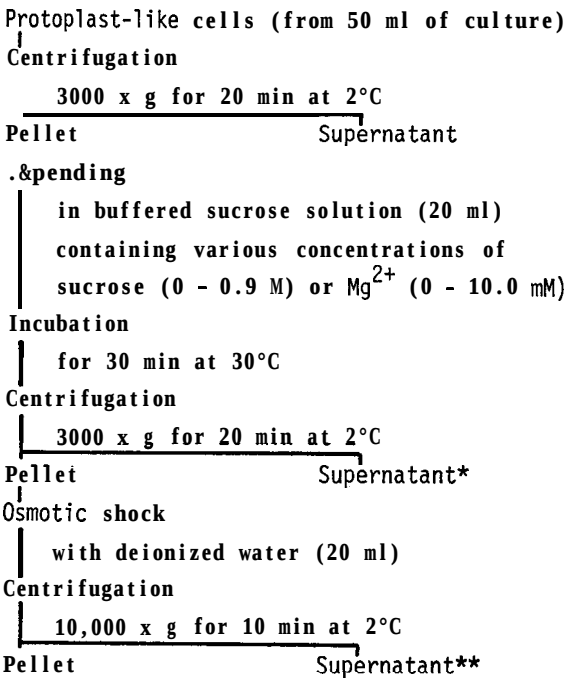

Fig. 6. Procedure to investigate the effect of sucrose or Mg^{2+} concentration on the stability of protoplast-like cells. The effect of sucrose or Mg^{2+} concentration was evaluated by measuring OD_{260} of the * and ** marked supernatants.

The adsorption rate on PLCs always lied on 5 to 8 % at multiplicity of infection (m. o. i.) of 5. Electron microscopy of the phage infected PLC culture exhibited that the constant adsorption of phages was depended upon a very small portion of contaminated cell wall fragments and whole cells. It was also clear that the adsorption rate decreased inversely to the number of added phage particles and was much rapidly than that of normal cells. This

Table 2. Adsorption. rate of bacteriophages on protoplast-like cells. Bacteriophages HM 2 and HM 3 were infected to protoplast-like cells with various multiplicity of infection, and then incubated for 15min at 30°C. Phage particles remaining in the supernatant fluid was assayed as described in the Materials and Methods. The numbers in the Table represent the per cent of adsorbed phage particles.

m.o.i.*	HM 2		HM 3	
	NC**	PLC***	NC	PEC
0.1	99.9	35. 1	96. 5	14.9
0. 5	99.9	24. 3	92.3	12.5
1. 0				8. 9
5. 0	99. 99.5 7	16.8 8.4	86. 85.7 a	5. 1

* Multiplicity of infection. ** Normal cells. *** Protoplast-like cells.

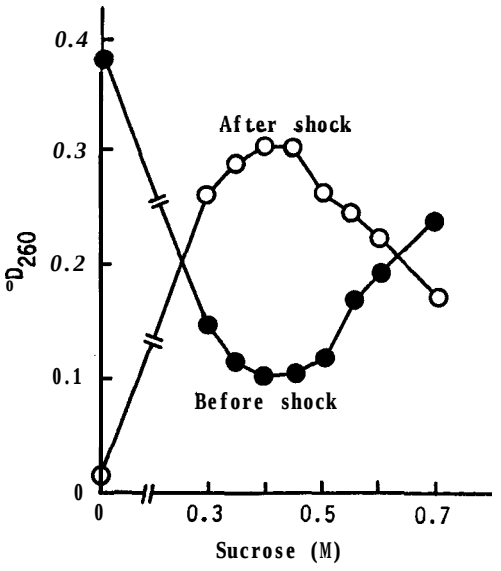

Fig. 7. Effect of sucrose concentration on the stability of protoplast-like cells. Experiment was carried out according to the procedure illustrated in Fig. 6. The lower absorbance before osmotic shock and the higher absorbance after osmotic shock indicate the better effect on the stabilization of protoplast-like cells.

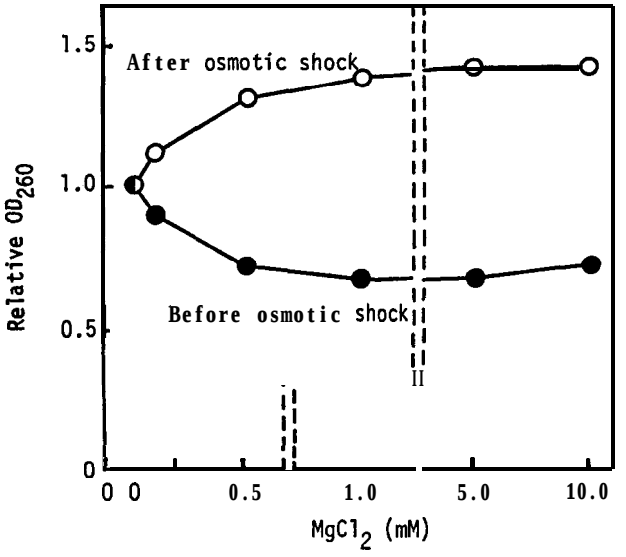

Fig. 8. Effect of Mg^{2+} concentration on the stability of protoplast-like cells. Experimental conditions were the same as Fig. 7, except that sucrose was substituted to $MgCl_2$.

indicates the existence of limited number of receptors. From these results, it is clear that PLCs are resistant against phage adsorption. These results also suggest that PLCs have no cell wall.

Biological properties of protoplast-like cell (PLC)

(1) *Growth of PLC*

Turbidity of the PLC culture increased by succeeding cultivation in osmotically stabilized liquid medium. However, when they were plated to form their colonies, 1.5×10^{2} colonies were only formed from 1.0 ml of PLC culture which contained about 2.5×10^{8} PLCs (microscopic count). In this experiment, a solid TYA medium containing 0.1-O. 4 M sucrose was used. However, so far as tested, it was impossible to improve the ability to form colony. This result indicates that PLCs are able to grow turbidmetrically but not be able to multiply. The increase in turbidity of PLC culture depended upon the volumetric growth of individual PLCs. However, we should like to continue the work on the regeneration of PLCs.

(2) *Macromolecular biosynthesis in PLC*

Macromolecules of growing PLCs were fractionated into DNA, RNA and

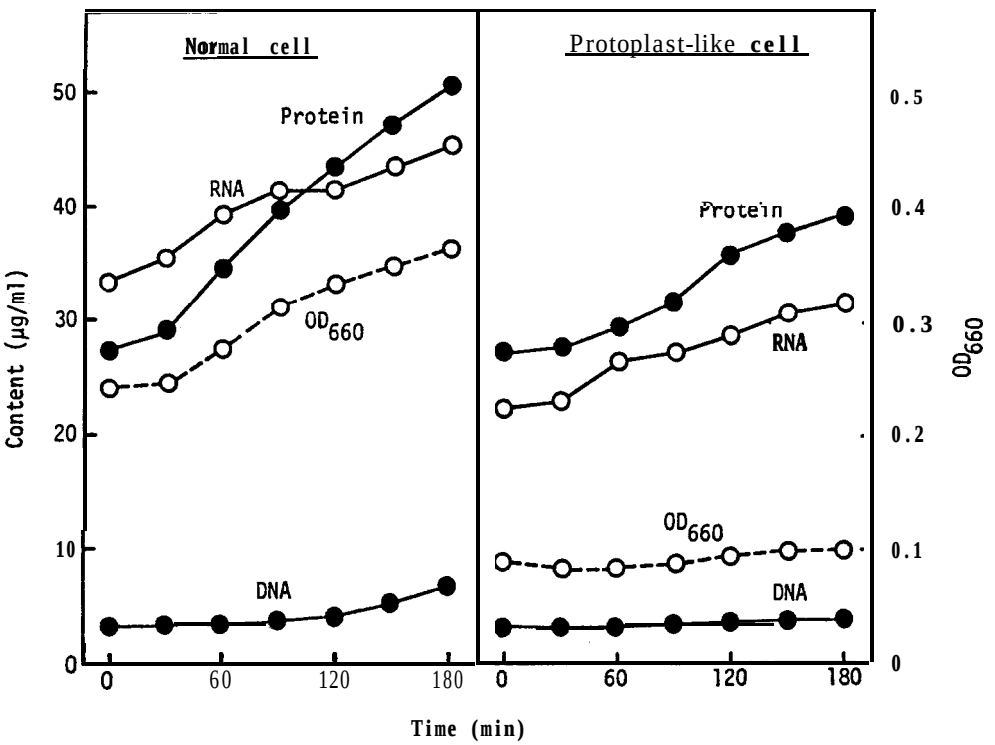

Fig. 9. Macromolecular biosynthesis in protoplast-like cells. Each macromolecule was fractionated according to the STS method, and the content was determined as described in Materials and Methods.

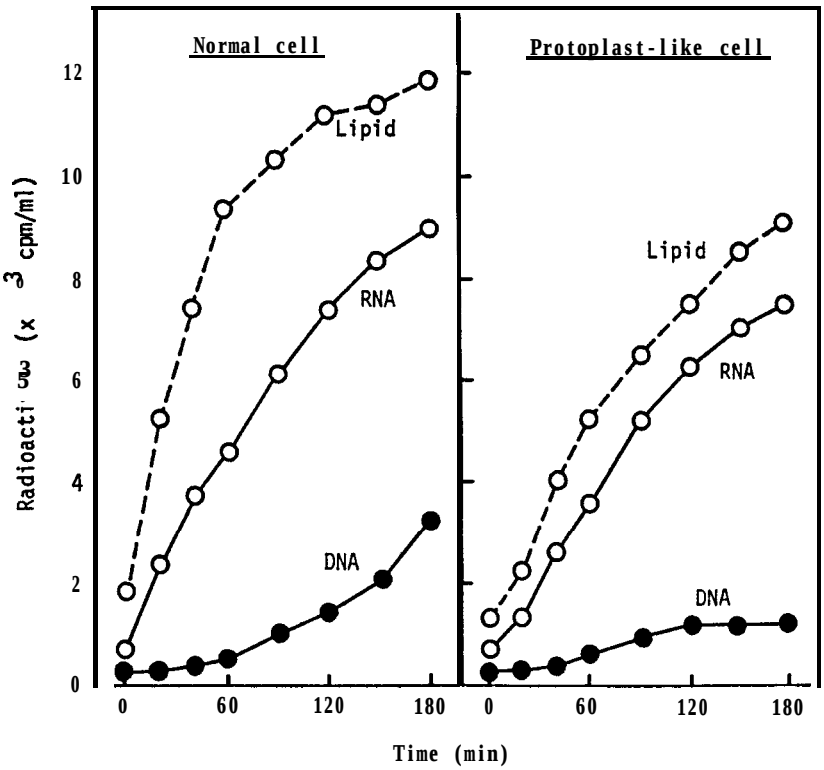

Fig. 10. Incorporation of ^{32}P-orthophosphoric acid into the macromolecules. Protoplast-like cells were cultivated in the presence of 2.5~ 10^{-3} mCi/ml of ^{32}P-orthophosphoric acid. Each macromolecule was fractionated according to the STS method. Radioactivity in each macromolecule was counted with a gas flow counter.

protein, and the content of each macromolecule was determined according to the methods described in Materials and Methods. As shown in Fig. 9, each macromolecular content increased with balanced manner for 2 hr. However, after that time, DNA content hardly increased, while other macromolecules of RNA and protein content continuously increased.

To confirm such manner of macromolecular synthesis in the PLCs, they were cultivated in the presence of ^{32}P-orthophosphoric acid and their macromolecules were radiolabelled. As shown in Fig. 10, DNA, RNA and lipid showed a balanced incorporation of radioisotope within 2 hr from the beginning of cultivation. After then, ^{32}P was hardly incorporated into DNA fraction, while it continuously incorporated into RNA and lipid fractions, as a good accordance with the result of above determination. Furthermore, to certify the weakening of DNA biosynthetic ability of PLCs at the later period of cultivation, ^{3}H-thymidine was added to PLC culture cultivated already for 3 hr. As shown in Fig. 11, little incorporation of ^{3}H into the PLCs was observed.

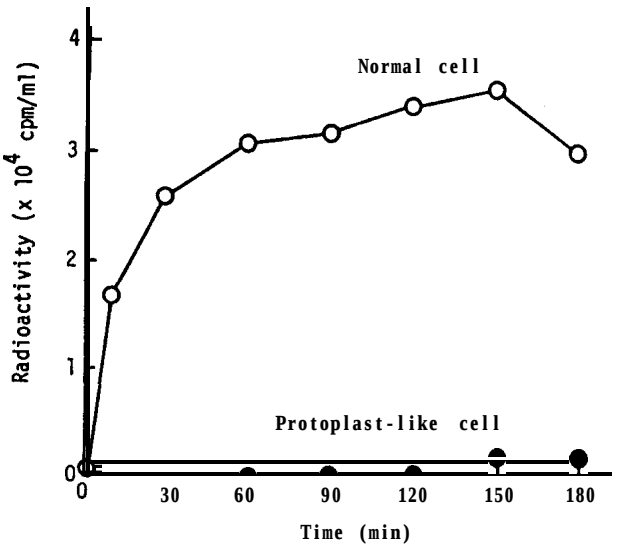

Fig. 11. Incorporation of ^{3}H-thymidine into protoplast-like cells. After 3 hr of cultivation, protoplast-like cells were exposed to methyl-^{3}H-thymidine (1. Ox 10^{-3} mCi/ml) and then cultivation was continued. Withdrawn protoplast-like cells were decomposed with N KOH and the radioactivity was counted with a scintillation counter.

From these **results,** it is clear that PLCs have two different manners of macromolecular biosynthesis : one is a balanced macromolecular biosynthesis during early period of cultivation, and the other is an unbalanced macromolecular biosynthesis, due to the weakened DNA synthesis, during late period of cultivation. PLCs may have a limited ability of their DNA synthesis. Thus, they can not produce their progeny cells, even when they are cultivated in the stabilizing liquid medium.

Table 3. Production of an inducible bacteriocin by protoplast-like cells. Protoplast-like cells were cultivated for 3 hr in the presence of various concentrations of mitomycin C. Activity of clostocin 0 liberated into the supernatant fluid was assayed by spot test. The activity was estimated as described in the Materials and Methods.

Mitomycin C (μg/ml)	Protoplast-like cells (unit/ml)	Normal cells (unit/ml)
none	—*	—
0.1	3-5	—
0.5	o-2	5-10
1.0	—	o- 2
5.0	—	—

* Bacteriocin was not produced.

Capability of protoplast-like cell (PLC) to produce bacteriocin and bacteriophage

Strain **Nl-4** can produce a phage tail-like bacteriocin clostocin 0 after treatment with low concentration of mitomycin C (Ogata *et al., 1972).* It is also infected by phages HM **2** and HM 3 (Hongo and Murata, 1965). Thus, experiments were carried out to understand whether or not PLCs can produce the bacteriocin and phages under the limited ability of DNA synthesis.

(I) *Induction of bacteriocin from PLC*

As shown in Table 3, PLCs produced clostocin 0 in the presence of 0.1 to 0.5 μg/ml of mitomycin C. Optimum concentration of mitomycin C for PLCs was at 0.1 μg/ml, while 0.5 μg/ml for normal cells. The size of liberation from PLCs was about 50 % of that from normal cells. This result indicates that PLCs are able to produce clostocin 0 as well as the parent cells. It is considered that the lack of cell wall on PLCs may be shifted to lower concentration of the drug for the production of clostocin 0 than that for the normal cells.

(2) *Transfection of PLC with isolated phage DNA*

Transfection experiment was performed with phage HM 2 DNA isolated. It was confirmed that there was no contamination of infectious phage particles in the DNA preparation. PLCs of two mutant strains (Nl-611 and Nl-504) of Nl-4, resistant mutants against phage HM 2 adsorption, were also used in this experiment to prevent the produced progeny phages from adsorption to the contaminated cell wall debris and whole cells.

As shown in Table 4, PLC culture of each strain produced infectious progeny phages (0.8 to 1.2×10^9 p.f.u/ml) by infection of isolated phage DNA. This productivity of progeny phages by transfected PLCs was less than 5 % of that of normal cells infected by intact phages. No phages were detected

Table 4. Transfection of bacteriophage DNA on protoplast-like cells. Infectious preparation of phage HM 2 DNA (0.2 ml) was added to the culture of protoplast-like cells (2 ml, about 2 x 10^8 cells/ml). The infected protoplast-like cells were cultivated for 3 hr in a stabilized TYA medium. After centrifugation (10,000$\times g$ for 10 min at 2°C), the number of active phage particles in the supernatant fluid was assayed by double layer plate technique. Strains of Nl-504 and Nl-611 are mutants from strain N1-4 and resistant against phage HM 2 adsorption. Their protoplast-like cells were prepared by the same method as strain Nl-4.

Experiment	Phage production
DNA only	−[c]
decomposed DNA[a]+PLC[b] of N1-4	−
DNA+normal cells of Nl-4	±[d]
DNA+PLC of Nl-4	+[e]
DNA+PLC of Nl-611	+
DNA+PLC of N1-504	+

a) Decomposed by treatment with 5 μg/ml of DNase. b) Protoplast-like cells. c) No production. d) As maximum, 10 p.f.u./ml of phage particles were detected. e) 0.8 to 1.2×10^9 p.f.u./ml of phage particles were detected.

from the PLC culture with DNase-treated DNA. This result indicates that PLCs can produce phage progenies as well as the normal cells. Less than 10 p.f.u./ml of phages were detected from normal cell culture with isolated DNA. This production of phage progenies may be due to the cells of competent state which were very rare concomitant in normal culture.

DISCUSSION

There are two ways in which the morphological changes in the conversion of the rod-shaped cells to protoplast-like cells (PLCs) take place, as shown schematically in Fig. 12. One occurs easily in the large pre-division cells, as well as protoplasts of *Escherichia coli* (Hahn and Ciak, 1957). The other occurs easily on the small post-division cells. The former is closely related to the process of morphological change beginning at the middle, while the other is related to the process of morphological change beginning at the pole.

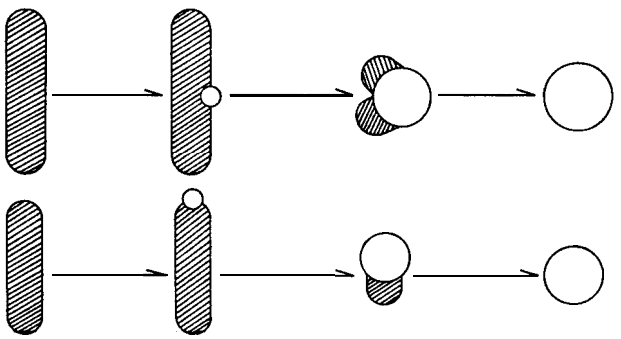

Fig. 12. Schematic representation of morphological changes in *Clostridium saccharoperbutylacetonicum* during sucrose-induced autolysis. Upper line indicates the morphological changes beginning at the middle of the cell which occur easily in the large pre-division cells. Lower line indicates the morphological changes beginning at the pole of the cell which occur easily in the small post-division cells. Shaded parts indicate the remaining parts of the original rod, while the bulbs indicate extruded cytoplasmic components.

It is clear that the clostridial cells were converted into PLCs by degradation of the rigid cell wall in the hypertonic sucrose solution. Cellular autolysin must be the only murolytic enzyme concerned in sucrose-induced autolysis. In many kinds of bacteria, it has been demonstrated that active autolysin is predominantly associated with the newly synthesized cell wall (Higgins et ***al.,*** 1970; Pooley and Shockman, 1970; Tomasz et ***al.,*** 1975), and is mainly located on the cell wall at the middle (septal site) or at a pole of the cell (Hughes and Stokes, 1971; Kawata and Takumi, 1970; Schwarz ***et al.,*** 1975). Those cellular zones are suggested as the sites of cellular autolysis of ***Lactobacillus*** *acidophilus* (Higgins ***et al,,*** 1973). In C. *saccharoperbutylacetonicum*, as well as *Clostridium perfringens* type A (Kawata and Takumi, 1970), the beginning site of degrada-

tion of the cell wall was also defined as the middle or a pole of the cell.

It is theoretically possible that bacterial cells are converted into protoplasts through morphological changes beginning at both the middle and one pole. However, the beginning site of morphological changes in ***E. coli*** seems to be either the middle or the pole of the cell. Cells of ***E. coli*** treated with penicillin were converted into the spheroplasts through the morphological change beginning only at the middle (Kaback, 1971). On the other hand, the cells autolysed in buffer were converted into the spheroplasts through the morphological change beginning only at one pole (Mohan ***et al.,*** 1965). In sucrose-induced autolysis, the clostridial cells were converted into PLCs through the morphological changes beginning both at the middle and one pole. The other morphological changes corresponded well with each case of lysis of ***E. coli*** (Hahn and Ciak, 1957; Kaback, 1971; Mohan ***et al.,*** 1965). We will report the role of autolysin and sucrose in the morphological change of the clostridial cells which occur during sucrose-induced autolysis in our next paper.

There has been an offer to call the osmotically fragile and microscopically spherical cells as "protoplast-like bodies" when detailed properties of them were obscure (Weibull, 1963). This offer was made to prevent the confusions among physically resemble other bacterial forms such as spheroplasts and L-forms. The spherical cells produced by sucrose-induced autolysis were named as protoplast-like cells (PLCs) according to the offer, and their properties were investigated to know whether or not they were real protoplasts.

Osmotic fragility has been known as a basic property of protoplasts (Martin, 1963; Weibull, 1963). PLCs were apparently fragile to osmotic shock. Electron microscopies on the produced PLCs were highly instructive that PLCs had no cell wall on their surface. The resistibility of PLCs against phages was also instructive to the absence of cell wall. It has been known that PLCs of strain No. 8 were also resistant against the adsorption of clostocin 0 (Ogata ***et al.,*** 1976).

Concerning with the macromolecular biosynthesis in protoplasts, there have been found two different manners. One is a balanced macromolecular biosynthesis as demonstrated by "autoplasts" of S. ***faecalis*** (Roth ***et al.,*** 1971). The other is an unbalanced biosynthesis as demonstrated on the protoplasts of ***B. megaterium*** (Weibull and Beckman, 1960) and yeast spheroplasts (Hutchison and Hartwell, 1967). The unbalanced macromolecular biosynthesis of the PLCs was due to the weak biosynthesis of DNA.

It is difficult to judge which manner of biosynthesis our PLCs belong to. The manner of PLCs was neither balanced nor unbalanced, because they exhibited a combined manner of biosynthesis; a balanced biosynthesis during the early period of cultivation and an unbalanced (weak) biosynthesis of DNA during the late period of cultivation. This manner of biosynthesis may indicate that there is some limit on the content of DNA in individual PLC. Generally, DNA biosynthesis in a bacterium is coupled with the formation of septum, and it is necessary to synthesize the septum for initiation of new DNA biosynthesis (Helmstetter and Cooper, 1968). The lack of cell wall (including septum) formation ability may give a limit to the succeeding biosyn-

Table 5. Sucrose- or NaCl-induced autolysis compared with other bacterial autolysis reported.

Bacteria	Inducer	Suspending fluid	Lysis time	Osmotic stabilizer	Final cell form	Conversion rate	Reference
C. saccharoperbutylacetonicum N1-4	0.35 M sucrose	TYA medium or 1/60 M phosphate buffer (pH 6.0)	40-60 min	(inducer)	protoplasts	99%	
	0.35 M NaCl	same as above	15-20 min	5% polyethylene glycol	protoplasts	80-90%	
C. *sporogenes* — [a]		0.2 M Tris-maleate buffer (pH 7.0)	18 hr	0.5-1.5 M sucrose	spheroplasts	— [b]	Galli and Huges(1965)
C. ***botulinurn*** —		***0.05 M*** phosphate buffer (pH 7.0)	2-3 hr	0.5 M sucrose	spheroplasts	SO-SO%	Kawata ***et al. (1968)***
B. cereus T —		0.05 M Tris-ammonium buffer (pH 7.5)	3 hr	16% polyethylene ***glycol***	spheroplasts	99%	Mohan ***et al. (1965)***
B. megaterium KM	cessation of aeration	peptone water medium	1-3 hr	15% sucrose	spheroplasts	50-80%	Kawata ***et*** al. (1961)
S. ***faecalis 9790***	—	***0.04*** M ammonium acetate buffer (pH 7.0)	3-6 hr	0.5 M sucrose	protoplasts	—	Roth ***et al.*** (1971)
E. coli B		0.4 M sodium acetate buffer (pH 6.0)	3 hr	1.6 M sucrose	spheroplasts	90%	Mohan ***et al. (1965)***

a) No specific induction mechanism of autolysis other than incubating the bacteria in buffer solution was involved in the experiments. b) Conversion rate was not clarified in the reports.

thesis of DNA in PLC.

PLCs succeeded some capabilities of parent cells such as bacteriocin and phage production. On the contrary, they lost self-multiplication and ability of phage adsorption due to the lack of cell wall. Judging from these properties of PLCs, they must be the living protoplasts (autoplasts).

Sucrose-induced autolysis and autoplasts of C. *saccharoperbutylacetonicum* are compared in Table 5 with other cases of bacterial autolysis and formed protoplasts or spheroplasts.

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