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Studies on the Surface Barriers of *Bacillus megaterium**

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SUMMARY

The cytoplasmic membrane of *Bacillus megaterium* is a permeability-barrier for glutamic acid and neutral red.

There is no possibility that glutamic acid is pooled between the cell wall and cytoplasmic membrane.

INTRODUCTION

Tomcsik (1952) defined a bacterial cell lacking a cell wall as a protoplast. Weibull (1953) established the isolation and characterization method of protoplasts and demonstrated that the protoplast membrane corresponds to the cytoplasmic membrane, which is the permeability barrier of the bacterial surface. Recently Weibull (1956) and Mitchell (1956) described methods for estimating semipermeability of the protoplast of *B. megaterium*.

In this paper we provide evidence for the existence of a permeability barrier in the cytoplasmic membrane of *B. megaterium* through experiments on the fate of assimilated free glutamic acid during protoplast formation and through vital staining of the protoplast.

Materials and Methods

Bacteria.—B. megaterium KM was used. Bacteria were grown in broth which was mechanically shaken at 37°. A 15 hour culture was harvested by centrifugation and washed twice with saline.

Assimilation of glutamic acid.—30 mg. (dry wt.) of washed cells were suspended in 100 ml. of medium G, of which chemical composition is shown in Table 1. After one hour incubation at 37°, cells were centrifuged and washed several times with saline to remove components of the medium. These cells were used as the glutamic acid containing cells.

Table 1. Chemical composition of medium G

$Na_2HPO_4 \cdot 12H_2O$	0.9 gm.	Na glutamate	1.7 gm.
KH_2PO_4	6.5 gm.	Glucose	10.0 gm.
NaCl	5.0 gm.	Distilled water	1,000 ml.
$MgSO_4 \cdot 7H_2O$	0.1 gm.	pH	5.5

* This paper was read at the 10th Meeting of the Kansai Branch of the Society of the Japanese Bacteriologists in October 1957, at Nagoya City University.

Glutamic acid decarboxylase.—A glutamic acid decarboxylase producing strain of *E. coli* was grown in 300 ml. of medium GL which was mechanically shaken at 37° for 8 hours.

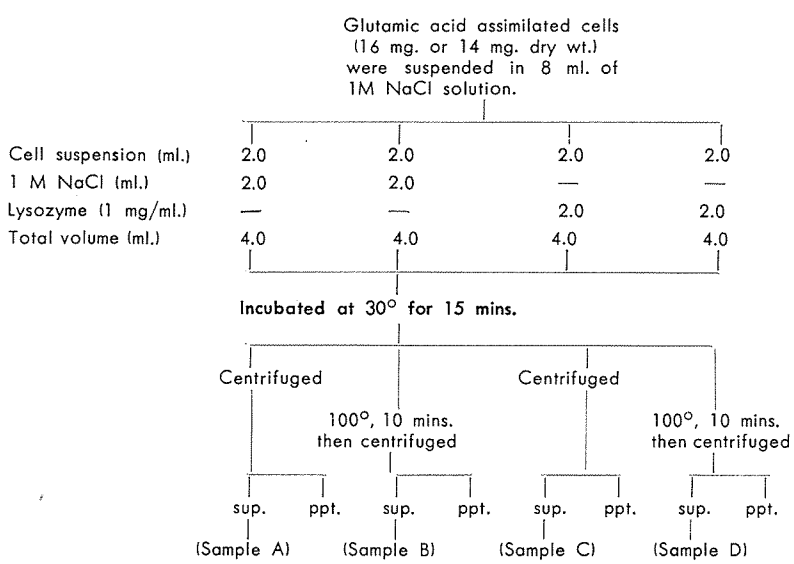
The chemical composition of medium GL was shown in Table 2. The culture was added to 1,000 ml. of this medium. After incubation at 30° for 16 hours, cells were centrifuged, washed twice with saline and dried in cold acetone (anhydrous). The dried cells were used as the decarboxylase preparation. In these experiments 3 mg. of decarboxylase was used for the determination of the glutamic acid contents of each sample. The enzyme preparation did not react with glutamine.

Table 2. Chemical composition of medium GL

Glucose	8.0 gm.	Na glutamate	10.0 gm.
$(NH_4)_2SO_4$	4.0 gm.	Pyridoxine	0.001 gm.
KH_2PO_4	8.0 gm.	Distilled water	1,000 ml.
Yeast extract (Daigo)	1.0 gm.	pH	5.0

Determination of glutamic acid.—The glutamic acid content of each sample was determined by Gale's manometric method (Gale, 1953) at 37°. Each Warburg vessel contained 1.0 ml. of the test solution and 1.5 ml. of 0.1 M acetate buffer (pH 4.0) in the main chamber, and 0.5 ml. of glutamic acid decarboxylase preparation (6 mg/ml.) in the side arm. Test solutions were prepared as described in Table 3.

Table 3. Method for preparation of the samples for the determination of glutamic acid



Isolation of protoplasts.—Protoplasts were isolated according to the method of Weibull (1953). In these experiments glutamic acid assimilated cells were treated

with crystalline lysozyme in a medium containing 1M NaCl. In this medium protoplast formation was complete within 15 mins. at room temperature.

Determination of nucleic acids.—In order to check disruption of the protoplasts, the nucleic acid content of each sample was determined in a Beckman spectrophotometer at 260 mμ.

RESULTS

The fate of glutamic acid during protoplast formation in 1M NaCl: If the assimilated glutamic acid accumulates between the cell wall and the cytoplasmic membrane, almost all the glutamic acid must escape when the cell wall is removed by lysozyme digestion. If assimilated glutamic acid accumulates inside the cytoplasmic membrane, glutamic acid should be retained within the protoplast when the cells become protoplasts.

While varying the concentration of NaCl in the medium during formation of protoplasts the turbidity of the protoplast solution was measured by its optical density at 550 mμ, and the nucleic acid concentration was estimated at 260 mμ. Thus the optimal concentration of NaCl to the protoplasts was determined. As lysis of the protoplast proceeded a decrease of the optical density at 550 mμ and an increase of the optical density at 260 mμ was detected.

It was found that the protoplasts of *B. megaterium* were stable in 1.0 M to 1.2 M NaCl.

When glutamic acid assimilated cells were incubated at 30° for 60 mins. the quantity of glutamic acid released increased even in 1.0 M NaCl medium. So it was essential to prepare the protoplast rapidly.

As shown in Table 4, 1.72 μM—2.9 μM of assimilated glutamic acid was found inside the bacterial cells (4 mg. dry wt.) and almost equal amounts of glutamic acid were found in the protoplasts. The glutamic acid concentration in the supernatant of the cells (Sample A) and of the protoplasts (Sample C) was neglisible.

It was concluded from these results that the assimilated glutamic acid inside the bacterial cells is not released during protoplast formation in 1M NaCl.

Table 4. Glutamic acid contents of samples prepared as shown in Table 3

Sample	Experiment 1*		Experiment 2**	
	Glutamic acid content	Optical density at 260 mμ	Glutamic acid content	Optical density at 260 mμ
A	0.23 μM	0.032 (1:10)	0.21 μM	0.030 (1:15)
B	1.95 (1.72)***	0.625 (1:10)	3.11 (2.90)***	0.890 (1:15)
C	0.23	0.017 (1:10)	0.23	0.037 (1:15)
D	1.98 (1.73)****	0.845 (1:40)	3.25 (3.02)****	0.900 (1:50)

* Experiment 1 was carried out using 3.5 mg. (dry wt.) of cells.

** Experiment 2 was carried out using 4.0 mg. (dry wt.) of cells.

*** The value indicates the net content of glutamic acid in the bacterial cells.

**** The value indicates the net content of glutamic acid in the protoplasts.

Permeability barrier of protoplast to neutral red:

Knaysi (1951) writes, "When a normal cell is suspended in a dilute solution of a dye, such as neutral red, the cytoplasmic membrane which has considerable affinity for dye remains colorless until the cell is severely injured or dies, indicating the role of the cell wall. The generally greater permeability of lipoid-soluble dyes may be attributed to their solubility in the substance of the cytoplasmic membrane."

If his concept is correct, the protoplast membrane could easily be stained by neutral red because it is naked and exposed to neutral red. In order to prove his hypothesis, we tried to stain protoplast in 0.2 M sucrose solution with neutral red and crystal violet.

Protoplasts were not stained by neutral red at as high a concentration as 200 p.p.m., but immediately took up crystal violet. Crystal violet can also stain normal living cells, since it is toxic for bacterial cells.

It is suggested from these results that the barrier for dye may be the cytoplasmic membrane rather than the cell wall.

DISCUSSION

When protoplasts of glutamic acid assimilated cells were prepared in a medium containing 10% polyethylene glycol (PEG) almost all the glutamic acid was released into the medium. However, there was no evidence of disruption of the protoplasts. It was found under the phase contrast microscope, that the protoplasts were of a typical spherical form and mass lysis of the protoplast was never observed. In addition, release of nucleic acid into the medium of the protoplasts was not observed.

At lower concentrations of NaCl release of glutamic acid was observed in parallel with that of nucleic acids. Therefore the phenomenon observed in 10% PEG differs from that in NaCl medium. The explanation is not yet known.

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