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# Spheroplasts of Colicine K Producing Escherichia coli K235 prepared by Leucozyme C and by Lysozyme

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SUMMARY

1. A fraction having the activity of leucozyme C was obtained by absorbing the neutral solution extracted from lyophylized guinea pig leucocytes with zymozan.

2. The ghosts of spherical bodies from *Escherichia coli* $K_{235}$ prepared with leucozyme C as well as lysozyme/EDTA still retained the stable colicine K O antigen and were agglutinated with somatic O antibody in the colicine K antiserum. Hence the spherical bodies prepared by leucozyme C were proved to be spheroplasts.

3. During spheroplast formation in *E.* coli $K_{235}$ by lysozyme/EDTA, the colicine K O antigen complex was released from the cell walls. On the contrary, leucozyme C released colicine K protein from the cell walls of the bacteria. Liberation of colicine K protein was directly demonstrated by incubation of leucozyme C with colicine K O antigen.

4. As colicine K protein was found in the spheroplasts prepared with leucozyme C or lysozyme/EDTA, it can be assumed that colicine K protein is synthesized inside the cytoplasmic membrane.

## INTRODUCTION

For several years various methods for spheroplast formation in Gram negative bacteria have been developed (Zinder *et al.*, 1956, Repaske, 1956). They have been shown to retain some cell wall components and the term, spheropalst, has been adopted in place of protoplast (Brenner *et al.*, 1958). It was reported from this laboratory, that leucozyme C of guinea pig leucocytes could convert normal *E. coli* B cells to spherical bodies in a sucrose controlled medium, but the cells changed to ghosts when sucrose was not added to the medium (Amano *et al.*, 1956). We called these spherical bodies protoplasts in the report, however the evidence of protoplasts in the strict sense was not brought to those spherical bodies.

In this work, the surface structure of the spherical bodies prepared by leucozyme C was reinvestigated using colicine K producing *E. coli* $K_{235}$ and compared the bodies with those of spheroplasts prepared with lysozyme/EDTA (Fraser *et al.*, 1957). The latter have already been shown to retain some cell wall components (Brenner *et al.*, 1958). As the somatic O antigen of colicine K producing *E. coli* $K_{235}$ $L^+OC^+$ was shown to bear colicine K activity by Amano, Goebel and Miller Smidth (1958), the colicine K O antigen complex was used to detect cell wall components for characterization of the spherical bodies. To gain further information on the distribution of the colicine K activity in bacterial cells, the

colicine K activity was assayed in every fraction during conversion of the cells to spherical bodies.

## MATERIALS AND METHODS

1. *Bacterial strains: E. coli* $K_{235}$ $L^+OC^+$ and *E. coli* $K_{235}$ $L^-OC^+$ : The colicinogenic strains used in this study were kindly given by Dr. Walther F. Goebel of the Rockefeller Institute.

*E. coli* $K_{235}$ $L^-OC^-$ : This non-colicinogenic mutant obtained from *E. coli* $K_{235}$ $L^-OC^+$ was originally isolated by one of the present authors in the Rockfeller Institute (Amano, Goebel and Miller Smidth, 1958).

*E. coli* B: This strain was employed for colicine K assay.

2. *Culture medium:* As already described, specially defined media were required for the successful conversion of Gram negative bacteria to spherical bodies and for colicine K production. After several trials, the authors found the following medium was the most favorable; polypeptone (Takeda Chemicals) 20.0g., NaCl 5.0 g., $Na_2HPO_4$ 5.0g., yeast extract (Daigoeiyo Chemicals) 5.0g. and $H_2O$ 1,000 ml.

3. *Cultural condition of E. coli* $K_{235}$ $L^+OC^+$ *for conversion of cells to spherical bodies:* 50 ml. of the medium was seeded with $5 \times 10^5$ cells of *E. coli* $K_{235}$ $L^+OC^+$ and incubated at 37°C. with moderate aeration. The cells were harvested at the concentration of $2 \times 10^8$ cells per ml.

4. *Colicine K assay*: The assay method used in this study was the method B described by Goebel, Barry and Shedlovsky (1956).

5. *Antigen*: Purified colicine K was also kindly sent by Dr. Walther F. Goebel.

6. *Colicine K antiserum*: Antisera to purified colicine K were obtained by injecting rabbits with a sterile solution of colicine K as previously described by Goebel and Barry (1958).

7. *Lysozyme spheroplasting*: The procedure for formation of spheroplasts by lysozyme was the same as that described by Fraser *et al.* (1957).

## EXPERIMENTAL

1. *A new method for purification of leucozyme C from guinea pig leucocytes:*

As the preparation of leucozyme C reported previously from this laboratory was too weak to cause formation of spherical bodies in a large volume of bacterial suspension, attempts were made to find a more efficient preparative method of leucozyme C.

100 mg. (equal to about $6 \times 10^8$ leucocytes) of lyophylized guinea pig leucocytes were added to 5.0 ml. of buffered saline (pH 7.0) and the mixture was stirred for 20 min. and centrifuged in the cold. Zymosan was added to the supernatant to a concentration of 10 mg. per ml. The mixture was incubated at O°C. for 10 min. with occasional shaking and centrifuged at 10,000 r.p.m. for 15 min. at O°C. The supernatant was used as the leucozyme C preparation. The bacterial cells harvested at a concentration of $2 \times 10^8$ cells per ml. were washed once with saline and resuspended in saline adjusting the optical density to $D_{5500} = 0.08$ in a Coleman junior spectrophotometer using an 8 mm. diameter cuvette. The leucozyme C activity was tested with this bacterial suspension. The contents of the tubes and the results are shown in Table 1.

As can be seen in the table, conversion to spherical bodies was complete in 60 min. in the presence of 17 per cent sucrose. On the other hand, bacterial cells changed to ghosts when sucrose was omitted.

The crude extract of lyophylized guinea pig leucocytes contained lysozyme equivalent to about 80 $\mu$g. per ml. of crystalline egg white lysozyme. However

Table 1. The activity of leucozyme C obtained by absorption with zymosan

| | Tube number | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Leucozyme C | — | 3.0 | 3.0 |
| M/30 phosphate buffer | 4.0 | 1.0 | 3.0 |
| 85% sucrose | 2.0 | 2.0 | — |
| Bacterial suspension | 4.0 | 4.0 | 4.0 |
| incubated at 37°C. and at following intervals observed with a phase contrast microscope | | | |
| 30 min. | — | ++ | +++ |
| 60 | — | +++ | * |
| 120 | — | +++ | |
| 180 | — | +++ | |

++ : Almost all of bacteria changed to spherical bodies.
+++ : Bacteria completely changed to spherical bodies.
* : Almost all of bacteria changed to an amorphous mass.

after zymozan treatment the lysozyme content decreased to 0.8-1.2 $\mu$g. per ml. This low concentration of lysozyme would play some minor role in the conversion to spherical bodies, because addition of egg white lysozyme to leucozyme C preparation enhanced the rate of conversion to spherical bodies as already reported in our previous paper (Amano *et al.*, 1956). However, the major role must be played by leucozyme C, because versene and tris buffer, which are indispensable for the lysozyme spheroplasting, were not employed in this experiment.

A leucozyme A-like substance, which changes the spherical bodies to ghosts in the presence of sucrose, could be eluted at pH 3.5 from the zymosan used in the process of leucozyme C purification. Formerly it was wrongly thought to be leucozyme A itself (Amano *et al.*, 1956), which was also found in this laboratory. However at present it is considered as a different entity from leucozyme A. Studies on this leucozyme A-like substance will be reported later.

2. *The amount of colicine K produced by bacteria in the log phase of growth:*

The colicine K produced by bacteria in the log phase of growth has already been estimated by Goebel, Barry and Shedlovsky (1956). To measure the amount of colicine K produced by the cells at the concentration of $2\times10^8$ cells per ml. in the medium used in this study, estimations were made on the whole culture and also on the supernatant.

The culture was centrifuged and the cells were resuspended in saline at a concentration of $5 \times 10^8$ cells per ml. The cell suspension and the supernatant were treated with several drops of chloroform and centrifuged after 15 min. The colicine K content was estimated according to the method B of Goebel, Barry and Shedlovsky (1956). Results are presented in Table 2.

As can be seen from the table, the supernatant obtained at the concentration of $2\times10^8$ cells per ml. did not contain an appreciable amount of colicine K and

Table 2. Colicine K content of *E. coli* $K_{235}$ $L^+OC^+$ at the concentration of $2\times10^8$ cells per ml.

| Tested sample | Dilution of sample | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 : 1 | 1 : 2 | 1 : 4 | 1 : 8 | 1 : 16 | 1 : 32 | 1 : 64 | 1 : 128 | 1 : 256 |
| Whole culture | 4 | 4 | 4 | 4 | 4 | 3 | 2 | 1 | ½ |
| Culture supernatant | 1 | ½ | ½ | 0 | 0 | 0 | 0 | — | — |

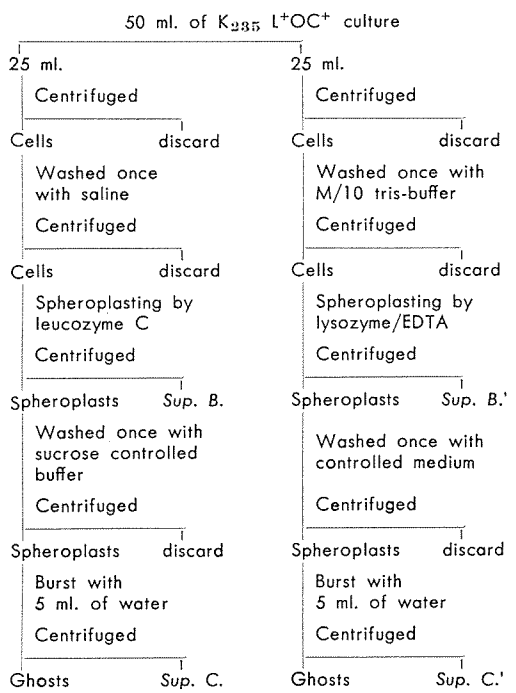
4: Complete inhibition of growth of *E. coli* B.
O: No inhibition of growth of *E. coli* B.

almost all of colicine K activity was still retained in the cells. This has already been reported by Goebel, Barry and Shedlovsky (1956). The colicine K content of the whole culture seemed sufficient to permit study of the distribution of colicine K activity in the cell.

3. *Characterization of ghosts:*

The colicine K activity of ghosts was assayed to see whether ghosts obtained from spherical bodies by osmotic shock still retained cell wall components. If the ghosts were composed only of the cytoplasmic membrane and did not contain cell wall components, colicine K activity should not be found in the ghosts fraction, and somatic O antigen of *E. coli* $K_{235}$ $L^+OC^+$ should not be detected by agglutination of the ghosts. In addition, the ghosts obtained from spherical bodies prepared by lysozyme should contain colicine K and somatic O antigen, because spherical bodies obtained by lysozyme/EDTA technique were already shown to be spheroplasts (Brenner *et al.*, 1958). Thus, the ghosts obtained from lysozyme spheroplasts served as controls in this experiment.

Fig. 1




Both methods for obtaining ghosts are presented in Fig. 1. The ghost fractions thus prepared were washed twice with 5.0 ml. of saline and resuspended in 5.0 ml. of saline. 0.1 ml. (10 mg.) of DNase solution, was added to each fraction and the mixture was incubated at 37°C. for 10 min. and then centrifuged. The precipitates were washed once with saline and each resuspended in 4.0 ml. of saline. Each suspension was divided into two equal portions. The one portion was heated at 100°C. for 10 min. Colicine was assayed in these four samples. The results are presented in Table 3.

Table 3. Colicine K activity of ghosts prepared by leucozyme C or lysozyme/EDTA

| Ghosts prepared with | | Dilution of ghosts suspension | | | | |
|---|---|---|---|---|---|---|
| | | 1 : 1 | 1 : 4 | 1 : 16 | 1 : 64 | 1 : 256 |
| Leucozyme C | non-heated | 4 | 3 | 2 | 1 | 0 |
| | heated at 100°C. for 10 min. | 4 | 2 | 2 | 0 | 0 |
| Lysozyme/EDTA | non-heated | 4 | 4 | 2 | 1 | 0 |
| | heated at 100°C. for 10 min. | 4 | 4 | 2 | 1 | 0 |

As can be seen from the table, ghosts prepared by leucozyme C and also by lysozyme/EDTA still retained colicine K activity. In addition, the colicine K activity in both kinds of ghosts was retained even after boiling at 100°C. for 10 min. The colicine K retained in both kinds of ghosts is bound with lipocarbohydrate complex, because it has already been reported that the colicine K O antigen complex is very active even after boiling at 100°C. for 10 min. (Goebel *et al.*, 1958). On the contrary, colicine protein was easily denatured by this treatment (Amano and Goebel, unpublished).

To confirm this, agglutination tests were made on both kinds of ghost suspensions with colicine K antiserum.

The ghost suspensions were concentrated by centrifugation. The dilutions of colicine K antiserum shown in Table 4 were made. One drop of ghost suspension was added to each tube. The tubes were incubated at 37°C. for 2 hrs. and subsequently at 4°C. overnight. The results are shown in Table 4.

Table 4. Agglutination of ghosts prepared with leucozyme C or lysozyme/EDTA in colicine K antiserum

| tested antigen | Dilution of antiserum | | | | | |
|---|---|---|---|---|---|---|
| | 1 : 200 | 1 : 400 | 1 : 800 | 1 : 1600 | 1 : 3200 | 1 : 6400 |
| Ghosts prepared with leucozyme C | ++ | ++ | + | ± | − | − |
| Ghosts prepared with lysozyme | +++ | ++ | + | + | ± | − |
| Living cells of $K_{235}$ $L^-OC^+$ | +++ | +++ | +++ | ++ | + | + |

As shown in the table, ghosts prepared by both leucozyme C and lysozyme were agglutinated by the antiserum, though their agglutination titers were somewhat lower than those of living cells of *E. coli* $K_{235}$ $L^-OC^+$. These agglutination were caused by somatic O antibodies and not by colicine K neutralizing antibodies, because it has already been reported (Amano, Coebel and Miller Smidth, 1958) that colicine K neutralizing antibodies in absorbed colicine K antiserum with non-colicinogenic *E. coli* $K_{235}$ $L^-OC^-$ were incapable of precipitating the colicine K O antigen complex or of agglutinating *E. coli* $K_{235}$ $L^-OC^+$.

This was reconfirmed by agglutination tests on both kinds of ghosts with colicine K neutralizing antibodies.

Colicine K antiserum was repeatedly absorbed with pellets of *E. coli* $K_{235}$ $L^-OC^-$ as described by Amano, Goebel and Miller Smidth (1958). The serum thus absorbed did not agglutinate *E. coli* $K_{235}$ $L^-OC^-$ or $L^-OC^+$ and did not precipitate colicine K O antigen, but still retained almost the same potency in neutralization experiments as the original antiserum. Using this absorbed antiserum, agglutination experiments were made of the two kinds of ghost suspensions. The results are presented in Table 5.

**Table 5. Agglutination of ghosts prepared with leucozyme C or lysozyme/EDTA in colicine K antiserum absorbed with *E. coli* $K_{235}$ $L^-OC^-$**

| Antigen | Dilution of antiserum | | | | | |
|---|---|---|---|---|---|---|
| | 1 : 100 | 1 : 200 | 1 : 400 | 1 : 800 | 1 : 1600 | 1 : 3200 |
| Ghosts prepared with leucozyme C | — | — | — | — | — | — |
| Ghosts prepared with lysozyme/EDTA | — | — | — | — | — | — |

As can be seen from the table, both kinds of ghosts could not be agglutinated. From these above results, it can be stated that the spherical bodies prepared by leucozyme C should also be termed spheroplasts.

4. *Distribution of colicine K activity in the cell:*

It was very peculiar that the lysates of spheroplasts prepared by either method contained appreciable amounts of colicine K activity. The fact led us to characterize the physicochemical properties of colicine K in every fraction listed in Fig.

**Table 6. Characterization of colicine K liberated from cell walls during protoplasting procedure**

| Spheroplasts prepared with | Tested samples | Tube number | Dilutions of Samples | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 : 1 | 1 : 2 | 1 : 4 | 1 : 8 | 1 : 16 | 1 : 32 | 1 : 64 | 1 : 128 | 1 : 256 |
| Leucozyme C | Sup. B (cell wall digest) | 1 | 4 | 4 | 4 | 3 | 2 | 1 | 1 | ½ | 0 |
| | | 2 | 1½ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Sup. C (Spheroplast lysate) | 1 | 4 | 3 | 2 | 1 | 1 | ½ | 0 | 0 | 0 |
| | | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lysozyme/EDTA | Sup. B' (cell wall digest) | 1 | 4 | 4 | 4 | 4 | 4 | 3 | 2 | 1 | 0 |
| | | 2 | 4 | 4 | 4 | 4 | 3½ | 2 | 2 | 1 | 0 |
| | | 3 | 4 | 4 | 4 | 3½ | 3 | 2 | 2 | 1 | 0 |
| | Sup. C' (spheroplast lysate) | 1 | 4 | 4 | 4 | 4 | 4 | 4 | 3½ | 2 | 1 |
| | | 2 | 1½ | 1 | ½ | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Tube number 1 : untreated control.
2 : heated at 100°C. for 10 min.
3 : treated with trichloracetic acid.

1. As already described by Goebel and Barry (1958), the colicine K O antigen complex was very stable when boiled at 100°C. for 10 min. and could not be precipitated by 10 per cent trichloracetic acid; on the other hand, colicine K protein was precipitated by trichloracetic acid and was labile when boiled at 100°C. for 10 min. These differences were adopted as criteria to identify colicine K O antigen and colicine K protein.

Leucozyme C in the cell wall digest (Sup. B in Fig. 1) was denatured at 60°C. for 10 min. and centrifuged. The supernatant was divided into three portions. The first was heated at 100°C. for 10 min. To the second, trichloracetic acid was added at a concentration of 10 per cent. The last served as a control. All the tubes were centrifuged and dialyzed against distilled water for 24 hrs. at 4°C. The dialyzed solutions were centrifuged and the supernatants were assayed for colicine K activity. The digest of cell wall by the lysozyme/EDTA method (Sup. B' in Fig. 1) was first dialyzed and then the dialyzed solution was divided into three portions but was not heated at 60°C. for 10 min. Further treatment was the same as that of Sup. B. To each 5.0 ml. of the spheroplast lysates (Sup. C and C') 0.1 ml. (0.4 mg.) of DNase solution was added, and the mixture was incubated at 37°C. for 10 min. The centrifuged supernatants were further treated as Sup. B. The results of colicine K assays are listed in Table 6.

Colicine K liberated from cells during the leucozyme C spheroplasting was inactivated by heating at 100°C. for 10 min. and precipitated by trichloracetic acid; on the other hand, colicine K liberated during lysozyme spheroplasting was very stable when heated at 100°C. for 10 min. and could not be precipitated by trichloracetic acid. Therefore leucozyme C liberated colicine K protein from cell walls during the spheroplasting process and the mode of action of leucozyme C is quite different from that of lysozyme/EDTA.

From the results of Sup. C and C' shown in Table 6, it can be seen that osmotic lysates of both kinds of spheroplasts contained colicine K protein. Since even lysozyme spheroplasts gave colicine K protein when they broken, it can be assumed that colicine K protein was synthesized inside the cytoplasmic membrane during the logarithmic growth phase of the bacteria.

5. *Effect of leucozyme C on the colicine K O antigen complex:*

In this experiment attempts were made to demonstrate the direct action of leucozyme C in liberating colicine K protein from the colicine K O antigen complex.

200 $\mu$g. of colicine K, dissolved in 4.0 ml. of 1:30 M phosphate buffer at pH 7.0 were added to 1.0 ml. of leucozyme C solution and incubated at 37°C. for 90 min. Leucozyme C

**Table 7. The effect of leucozyme C on colicine K O antigen complex**

| | | Dilution (colicine K content in control sol.) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 40$\gamma$/ml. | 20$\gamma$/ml. | 10$\gamma$/ml. | 5$\gamma$/ml. | 2.5$\gamma$/ml. | 1.25$\gamma$/ml. |
| Leucozyme C treatment | non-heated | 4 | 4 | 4 | 4 | 3 | 2 |
| | heated at 100°C. for 10 min. | 2 | 1 | ½ | 0 | 0 | 0 |
| Control | non-heated | 4 | 4 | 4 | 4 | 4 | 3 |
| | heated at 100°C. for 10 min. | 4 | 4 | 4 | 3½ | 3 | 2 |

was denatured by heating at 60°C. for 10 min. and centrifuged. The supernatant was dialyzed against distilled water in the cold for 24 hrs. As a control, the same solution (4.0 ml.) of colicine K was added to 1.0 ml. of saline and treated in the same way. Each of the dialyzed solution was divided into 2 portions. One was heated at 100°C. for 10 min. All four samples were assayed for colicine K activity. The results are presented in Table 7.

As can be seen from the table, leucozyme C could liberate colicine K protein from the colicine K O antigen complex.

## DISCUSSION

It is well known that the cell walls of Gram negative bacteria are mainly composed of a lipocarbohydrate protein complex and those of lysozyme sensitive Gram positive bacteria are mainly composed of mucopolysaccharides. The spherical bodies prepared by lysozyme from Gram negative and Gram positive bacteria seem to be different in their surface structures. According to Brenner *et al.* (1958), the spherical bodies of *E. coli* prepared by the lysozyme/EDTA method still retain much lipocarbohydrate. The surface structure of the spherical bodies prepared with leucozyme C had not yet been characterized prior to this work. The present study showed that the spherical bodies prepard with leucozyme C should also be termed spheroplasts.

From studies on the physicohemical properties of the colicine K liberated from the cell walls during spheroplast formation, the mode of action of leucozyme C was elucidated. Leucozyme C liberated the colicine K protein from the cell walls but not in the form of colicine K O antigen, which is liberated by lysozyme during spheroplast formation. The liberation of colicine K protein by leucozyme C was directly shown by incubating leucozyme C with the colicine K O antigen complex. The possible enzymatic nature of leucozyme C is not yet known. However the authors are inclined to regard it as some lipolytic enzyme. If this were the case, leucozyme C would play another important role, the detoxification of endotoxin. This problem is now being studied.

During the study of distribution of colicine K activity in the cells, colicine K protein was found in the osmotic lysates of spheroplasts prepared by leucozyme C and lysozyme/EDTA. It is very interesting that colicine K protein was produced in the cytoplasm of the bacterial cells. Probably, colicine K protein synthesized in the cytoplasm would combine with somatic O antigen in the cytoplasmic membrane, where somatic O antigen will be synthesized.

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