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NATURE OF SPHEROPLASTING AGENT, LEUCOZYME C, IN GUINEA PIG LEUCOCYTES*

MAKOTO OZAKI, YASUSHI HIGASHI and TSUNEHISA AMANO

Department of Bacteriology, Osaka University Medical School
Department of Immunology, Research Institute for Microbial Diseases, Osaka University, Osaka

AKIO MIYAMA and SHUZO KASHIBA

Department of Bacteriology, Nara Medical College, Kashiwara, Nara
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SUMMARY The spheroplasting activity of the extract of guinea pig polymorphonuclear leucocytes on *Escherichia coli* B and other Gram-negative bacteria in sucrose medium was studied. The active substance, leucozyme C (LEC), was found in the microsomal fraction of the leucocytes and could not be solubilized. Its activity was destroyed by digestion with trypsin or phosphatidase A.

The spheroplasting activity of this substance was shown to require lysozyme. Divalent cations and dicoumarol were found to inhibit the activity, and ADP, ATP and other nucleoside triphosphates activated the reaction. The possible mechanism of the spheroplasting activity of LEC plus lysozyme is discussed in relation to metallic ion chelation by LEC and to the structure of the bacterial cell wall.

INTRODUCTION

In 1956, " leucozyme C," which causes the cells of *Escherichia coli* B to become spheroplasts in a sucrose controlled medium, was found in this laboratory in an extract of lyophilized guinea pig leucocytes (AMANO *et al.*, 1956 a). The principal characteristics of leucozyme C described were as follows. 1) It could be extracted by saline alone from lyophilized leucocytes but not from native ones and it was partially purified by isoelectric precipitation at pH 5.2. 2) It was relatively heat-labile, and its activity was enhanced by lysozyme. 3) The cell walls of *E. coli* B could be lyzed by leucozyme C alone or together with lysozyme.

As the partially purified preparation of leucozyme C, obtained by isoelectric precipitation at pH 5.2, contained only a trace of lysozyme, its spheroplasting activity was presumed to be distinct from that of lysozyme, and some kind of macromolecule was postulated as the substrate of leucozyme C, and this would also play a role in sustaining the rigidity of the bacterial cell wall together with the substrate of lysozyme. In this way, the enhancing effect of

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lysozyme on leucozyme C activity could be explained.

However, WEIDEL (1960) found that the rigidity of the bacterial cell wall is maintained exclusively by mucopeptide, which is the substrate of lysozyme, and that the cell wall of Gram-negative bacteria contains somatic O antigen and lipoprotein in addition to mucopeptide. These results forced us to reject our previous explanation and to reinvestigate the nature of leucozyme C and the role of traces of lysozyme in the partially purified preparation of leucozyme C. This paper reports results of this work.

MATERIALS AND METHODS

1. *Bacterial strains*

E. coli B was used to estimate the activity of leucozyme C. The following strains were used in tests on the sensitivity to leucozyme C: *Salmonella typhosa* strains 6S, Watson V, O–901 W, and PR58, *Salmonella hirschfeldii*, *Salmonella ballerup*, *Salmonella rostock*, *Salmonella moscow* 27, *Salmonella blegdam* 22, *Salmonella gallinarum* 416, *Salmonella pullorum* 971, *Salmonella enteritidis* 891, *Salmonella choleraesuis* 1348, *Salmonella choleraesuis* specific rough 1535, *Salmonella choleraesuis var.* Kunzendorf 1350, *Salmonella typhisuis* 1347, *Shigella flexneri* 2a, *Shigella sonnei* E90, *Klebsiella pneumoniae* ST-101, *Aerobacter aerogenes* B-1, *Proteus vulgaris* KS, *Serratia marcescens* X-100, *Vibrio cholerae* Inaba, *Vibrio tyrogenes* Shin-Deneke, *Neisseria gonorrhoeae* NG-105, *Staphylococcus aureus* Terashima, *Diplococcus pneumoniae* DP-3, *Streptococcus* pyogenes J-14A 7, *Bacillus subtilis* NRRL B-558, *Bacillus megaterium* KM. These bacterial strains were from the type culture collection of this institute. *E. coli* B, *E.coli* K235 L^+O and L^-O were from the stock collection of this laboratory.

2. *Culture media*

Y-medium consisted of polypeptone (Daigo Eiyo Chem. Co., Japan) 20 g, yeast extract (Daigo Eiyo Chem. Co.) 3 g, $Na_2HPO_4 \cdot 12H_2O$ 5 g, NaCl 5 g, H_2O 1000 ml (pH=7.2), and nutrient agar consisted of polypeptone 10 g, meat extract (Mikuni Kagaku Chem. Co., Japan) 10 g, NaCl 5 g, agar (Daigo Eiyo Chem. Co.) 15 g, H_2O 1000 ml.

3. *Bacterial suspensions*

E. coli B cells were grown in Y-medium with shaking at 37°C and harvested in the logarithmic growth phase. Cells were washed with M/15 phosphate buffer (pH 7.5) and suspended in the same buffer. The bacteria used in sensitivity tests were harvested from nutrient agar slants of overnight cultures and suspended in the same way.

The optical density of bacterial suspensions was read in a Coleman junior spectrophotometer at 550 mμ, using Model 14–302A cuvettes.

4. *Preparation of leucozyme C*

Guinea pigs were given an intraperitoneal injection of 5~10 ml of 5 per cent of sterile meat extract (adjusted to pH 7.2 with NaOH). After 16~18 hours the peritoneal exudates were collected in citrate-saline (NaCl 7.5g, Na-citrate 5 g, H_2O 1000ml) and filtered through four layers of cheesecloth. The leucocytes in the exudate were precipitated by centrifugation at low speed and washed once with citrate-saline and then with 0.4 per cent NaCl by centrifugation. The packed leucocytes were suspended in water and were lyophylized, unless they were to be used for preparation of subcellular fractions. To 1 g of leucocyte powder, 50 ml of M/15 phosphate buffer (pH 7.5) was added and the mixture was homogenized in a glass Potter-Elvejhem homogenizer in an ice-water bath. The homogenate was centrifuged for 15 min at 12,000 rpm, using the No. 2 head of Kubota refrigerated centrifuge. The supernatant was used as the preparation of leucozyme C. The supernatant was centrifuged again for 1 hr at 40,000 rpm using the No. 40 head of a Spinco L centrifuge. The pellets were suspended in 20 ml of distilled water in a Toyo Kagaku Kiki 20 kc sonic oscillator. The suspension was kept in ice-water and used as the LEC preparation. Although the LEC fraction still contained a trace of lysozyme, the term LEC activity is used here for the activity of the particles which made the bacterial cells sensitive to lysozyme.

5. *Preparation of subcellular fractions*

The procedures used were essentially the same as those described by COHN and HIRSH (1960). The packed leucocytes, collected as described above, were suspended in 0.34 M sucrose containing 0.002 M EDTA and homogenized with a magnetic stirrer

until 85 per cent cell breakage was obtained. The procedures used for separating the nuclear, granular, "mitocondrial" and microsomal fractions of the homogenate are described in the Results. Each precipitate was suspended in distilled water and sonicated and then dialyzed against distilled water to remove the added EDTA. The insoluble residues were removed by centrifugation.

6. *An E. coli B cell wall preparation was prepared as described by* BROWN *and* KOZLOFF (1957)

7. *For protein determination, the Biuret method was used*

8. *For determination of sialic acid, the method of* WARREN (1957) *was used*

9. *Chemicals*

All the reagents used were of analytical reagent grade. Crystalline lysozyme was supplied by Dr. S. SHINKA of this laboratory. "Habu" (*Trimeresurus flavoviridis*) venom was given by Dr. T. KUBO of this institute. Trypsin was a product from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Soybean trypsin inhibitor was from Nutritional Biochemical Corporation (Cleveland, U.S.A.). Pronase was from Kaken Kagaku Kogyo (Tokyo, Japan). ATP was from Schwarz Bioresearch Inc. (New York, U.S.A.). ADP, AMP, CTP, GTP and UTP were obtained from Sigma Chemical Company (St. Louis, U.S.A.). Dicoumarol was purchased from Tokyo Kasei Co., Ltd. (Tokyo, Japan). Colominic acid was kindly supplied by Dr. G. T. BARRY.

RESULTS

1. *Necessity of lysozyme for the activity of leucozyme C*

To see whether lysozyme is indispensable for the spheroplasting activity of leucozyme C, the lysozyme was removed completely from the leucozyme C preparation by gel-filtration. Fifteen ml of leucozyme C preparation was applied to a column (4 × 25 cm) of Sephadex G-75 in 0.05 M phosphate buffer and eluted with the same buffer. The first protein peak contained very weak spheroplasting activity and almost no detectable lysozyme activity. The peak of lysozyme activity was between the first and second peaks in the absorbancy at 280 mμ. The fractions of the first peak were pooled and again filtered through another column. As shown in Fig. 1, only the first peak was seen at the end of the void volume of the column and it was completely devoid of spheroplasting activity. However, when 0.1 μg/ml of lysozyme or the pooled lysozyme fraction was added to it, the spheroplasting activity was recovered. Thus lysozyme is indispensable for the spheroplasting activity of leucozyme C. However, the spheroplasts formed were not very stable in sucrose medium.

To obtain a leucozyme C preparation giving the stable spheroplasts in cooperation with lysozyme, attempts were made to purify leucozyme C, and this was achieved by ultracentrifugation. The first component, obtained by the second gel-filtration treatment, was centrifuged at 100,000 × *g* for 1 hour and the precipitate was resuspended in distilled water by sonication. This suspension was found to have stable spheroplasts. After centrifugation the supernatant contained spheroplast-destroying activity and this agent was tentatively named leucozyme Aα. Its activity was lost on heating the preparation at 60°C for 1 hour at neutral pH. This is in contrast to leucozyme A which is heat stable (AMANO *et al.*, 1956 b). The latter can be extracted from guinea pig leucocytes by acid, and it was tentatively named leucozyme Aβ.

To show the effect of the addition of lysozyme to lysozyme-free LEC, viable counts were made by plating samples after destroying the spheroplasts formed with the buffer used for dilution. The experimental conditions are shown in Fig. 2. In the three control tubes, the lysozyme, lysozyme-free LEC, and lysozyme together with lysozyme-free LEC respectively were replaced by the same volume of buffer. At intervals, samples were taken, and the ratio of spheroplasts to normal cells were calculated under a phase contrast microscope and also viable counts were made. As can be seen in Fig. 2, the cells of *E. coli* B were

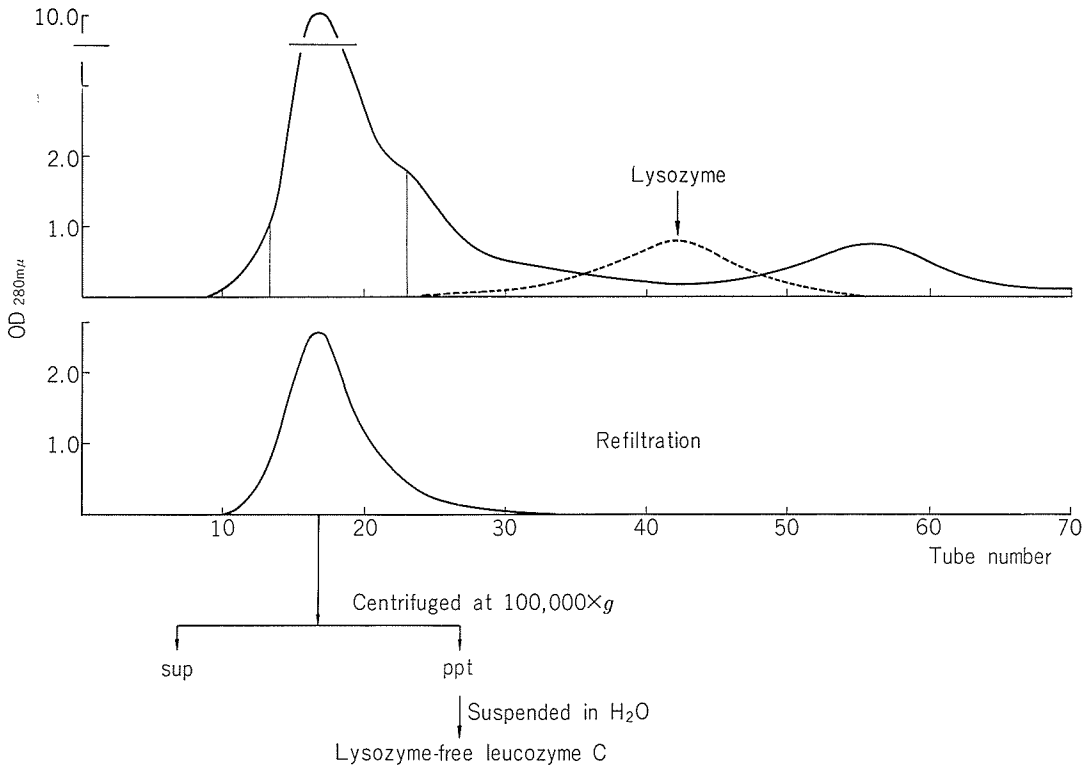

FIGURE 1 Gel-filtration of leucozyme C preparation.

changed to osmotically labile spheroplasts when LEC was added with lysozyme.

Though no data need be given, lysozyme-free LEC had no lytic effect on the mucopeptide of *E. coli* B prepared according to the method of MANDELSTAM (1961), while lysozyme digested it.

From these results it can be assumed that leucozyme C requires lysozyme for its spheroplasting activity and that the active particles of the LEC fraction render the cells of *E. coli* B sensitive to lysozyme.

2. *Kinetic studies on LEC activity*

To obtain information on the mode of action of LEC, a kinetic study was made and the effects of varying concentrations of LEC with a constant amount of lysozyme during incubation were investigated. The contents of the tubes were the same as those shown in Table 1, and there were four or five tubes of identical composition in each series. One tube from each series was taken out at intervals during incubation, and the spheroplasts formed were lyzed by adding 3 drops of 10 per cent sodium deoxycholate, the optical density being read

TABLE 1 *System for assay of LEC activity*

Bacterial suspension	OD = 1.0	2.0 ml
Sucrose	50 %	1.5
Lysozyme	100 μg/ml	0.5
LEC		0.5*
Phosphate buffer (or tris-buffer)	M/15 (M/20)	2.5*

* This volume was changed according to the LEC activity, but the total volume of the tube was always adjusted to 7.0 ml by varying the buffer volume. The incubation time was usually 30 min.

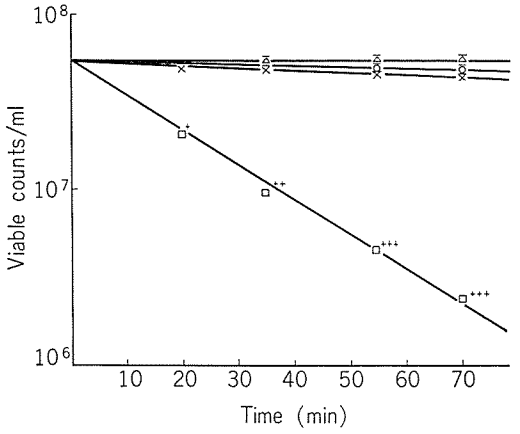

FIGURE 2 Effect of lysozyme on spheroplast formation by LEC.

□——□ Complete reaction mixture: LEC 0.1 ml, lysozyme (100 μg/ml) 0.1 ml, sucrose (50 per cent) 0.4 ml, bacteria (5×10^8/ml) 0.2 ml, 0.1 M phosphate buffer 1.2 ml

○——○ No lysozyme

×——× No LEC

△——△ No lysozyme and LEC in reaction system

− Indicates no spheroplast formation

+ Indicates that about 1/3 of bacteria were converted to spheroplasts

++ Indicates that about 3/4 of bacteria were converted to spheroplasts

+++ Indicates that all the bacteria were converted to spheroplasts

before and after this treatment. The difference between these values was assumed to be the optical density of the spheroplasts formed. The results are shown in Fig. 3. The difference between the initial optical density and that at the plateau was plotted against the amount of LEC added, as shown in Fig. 4. The results in Fig. 4 indicate that multiple hits of LEC are required for formation of spheroplasts in the presence of excess lysozyme. No conclusion can be drawn with regard to the turn over of LEC. Subsequently, the activity of LEC was estimated by the difference in optical densities before and after desoxycholate treatment.

3. *Adsorption of LEC on the cell walls of E. coli B*

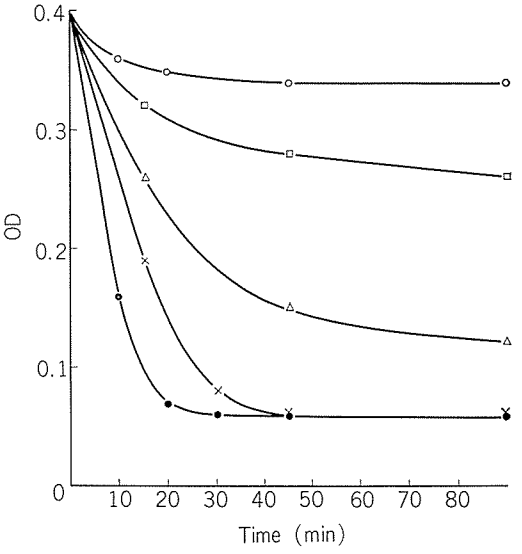

FIGURE 3 Spheroplast formation with various concentrations of LEC.

Various amounts of LEC preparation were added.

●——● 2.0 ml ×——× 1.0 ml

△——△ 0.5 ml □——□ 0.25 ml

○——○ Control

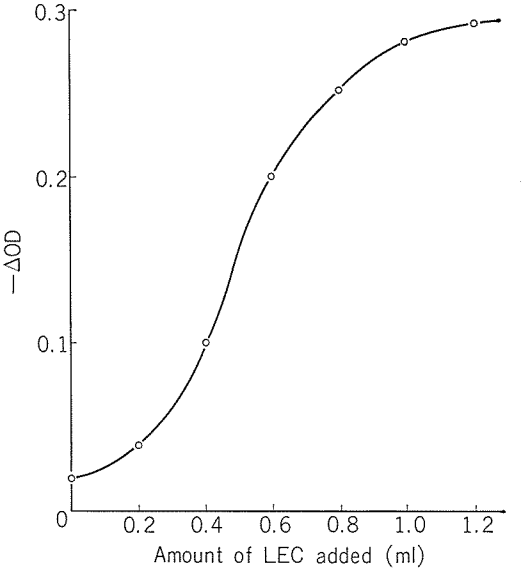

FIGURE 4 Relationship between LEC concentration and reduction of optical density.

If LEC is an enzyme attacking a component of the cell wall other than mucopeptide and turns over very quickly, it will not be adsorbed by the cell wall to any appreciable extent. To confirm this, 0.5 ml aliquots of LEC preparation were added to 1.5 ml of various concentrations of cell walls of *E. coli* B, and the tubes were left for 10 min at room temperature. After centrifugation at 12,000 rpm for 20 min the LEC activity remaining in the supernatant was assayed in the presence of lysozyme. The results shown in Fig. 5 indicate that LEC was adsorbed by the cell walls of the sensitive organism, *E. coli* B. However, no information was obtained with regard to its turn over. In the adsorption, 150 μg of the 600 μg of the added protein per ml was adsorbed by addition of 10 mg dry weight of cell walls. As the amount of cell walls added corresponds to twice the highest amount used in the experiments of Fig. 5, the active component, LEC, must represent less than 150 μg of protein.

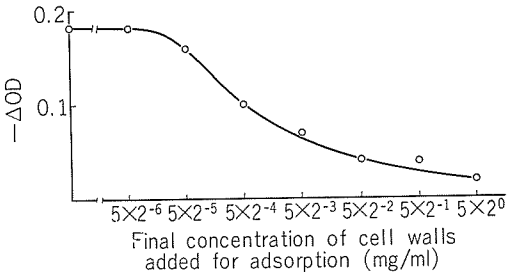

FIGURE 5 Adsorption of LEC activity on *E. coli* B cell walls.

4. *Action of LEC on various bacteria*

The antibacterial spectrum of LEC was examined. Almost all Gram-negative bacteria were sensitive to LEC together with lysozyme, as shown in Table 2. The exceptions among

TABLE 2 *Antibacterial spectrum of LEC*

		Conversion to spheroplast
Gram-negative	*Escherichia coli* B	+
	Escherichia coli K235L^{+}O	+
	Klebsiella pneumoniae ST-101	++
	Shigella sonnei E90	++
	Shigella flexneri 2a	++
	Serratia marcescens X-100	+
	Aerobacter aerogenes B-1	++
	Vibrio tyrogenes Shin-Deneke	+++
	Vibrio cholerae Inaba	++
	Neisseria gonorrhoeae NG-105	+++
	Salmonella typhosa (all strains tested)	−
	All other *Salmonella* strains tested	+
	Proteus vulgaris KS	−
	Escherichia coli K235 L^{-}O	−
Gram-positive	*Staphylococcus aureus* Terashima	−
	Streptococcus pyogenes J-14A 7	−
	Diplococcus pneumoniae DP-3	−
	Bacillus subtilis NRRL B-558	−
	Bacillus megaterium KM	−

− Not sensitive
\+ Slightly sensitive
++ Moderately sensitive
+++ Very sensitive

Gram-negative bacteria were *Salmonella typhosa*, *Proteus vulgaris* and *E. coli* K235 L^-O (capsular L-antigen negative, opaque, and colicine K and X producing). However, the L^+O variant of *E. coli* K235 was sensitive. With regard to the insensitivity of *Salmonella typhosa*, this has no relationship with the Vi-antigen, because Vi-antigen bearing *Salmonella paratyphi* C and *Salmonella ballerup* were sensitive whereas Vi-antigen bearing strains of *Salmonella typhosa* were insensitive.

From the difference in behavior with the two variants of *E. coli* K235 it seemed that LEC is an enzyme which hydrolyzes colominic acid which is the main component of the capsules of the L^+O variant. Therefore, the hydrolysis of colominic acid by LEC was examined by determining the sialic acid liberated (WARREN, 1959). However, negative results were obtained.

5. *Distribution of LEC in subcellular fractions*

Since the activity of LEC can be precipitated by centrifugation at 100,000 $\times g$ for 1 hour, it must be retained in some particulate fraction. To study this particulate fraction, subcellular fractions were prepared according to the method of COHN *et al.* (1960) and their LEC activity was examined.

Guinea pig leucocytes were washed with distilled water and suspended in 120 ml of 0.34 M sucrose containing 0.002 M EDTA at a cell density of $3\sim5 \times 10^7$ per ml. The cells were disrupted by a magnetic stirrer until 85 per cent of the cells were disrupted. The homogenate was centrifuged successively at 400 $\times g$ for 5 min, 8,200 $\times g$ for 10 min, 13,000 $\times g$ for 25 min and 100,000 $\times g$ for 60 min. The first, very sticky precipitate (Fr. N) contained nuclei and intact cells. The second contained granules and mitochondria (Fr. GM), the third mitochondria (and probably " lysosomes " if any) (Fr. ML), and the fourth contained microsomes (Fr. MC). Each of these precipitates was resuspended in 10 ml of distilled water and sonicated, and the suspensions were dialyzed against distilled water to remove EDTA. The dialysates were clarified by centrifuging at 6,000 rpm for 10 min. The LEC activity of 0.5 ml of each supernatant was estimated in the presence of excess lysozyme (7 μg per ml final concentration) as described above, and the activities of fractions N, GM, ML and MC were as follows: $-\Delta$OD =0.27, 0.02, 0.05, 0.25, respectively. The N and MC fractions were found to have LEC activity. The LEC activity found in fraction N can be explained by assuming that some of the activity was derived from the unbroken cells and some from the microsomal particles adsorbed onto the sticky nuclei.

To confirm this, the following experiments were performed. Fraction N, described above, was digested by DNase (40 μg per ml final concentration) and then fractionated as shown in Fig. 6. Both microsomal fractions (MC-1, MC-2) and the nucleus fraction (N-2) were active, but the three " lysosome " fractions (ML-1, ML-2 and ML-3) were inactive, as shown in Table 3. The activity found in the nucleus fraction may have been derived from the intranuclear particles in addition to the two other possibilities described above. However, as the nucleus fraction contained 5- or 7-fold more protein than the microsome fractions, the microsome fraction can be regarded as the main fraction with LEC activity.

The separation of ribosomes from fragments of endoplasmic reticulum and cell membranes was attempted using deoxycholate and centrifugation. However the results could not be evaluated because deoxycholate could not be removed completely and the deoxycholate remaining destroyed the spheroplasts formed and hence disturbed the assay of LEC activity. Moreover, attempts to solubilize LEC activity were unsuccessful.

To see whether LEC activity is also present in the microsomal fractions of other organs of the guinea pig, the liver, spleen and lung were examined but only very weak activity was found in the lung and none in the other tissues. Ehrlich ascitic tumor cells were also examined with negative results.

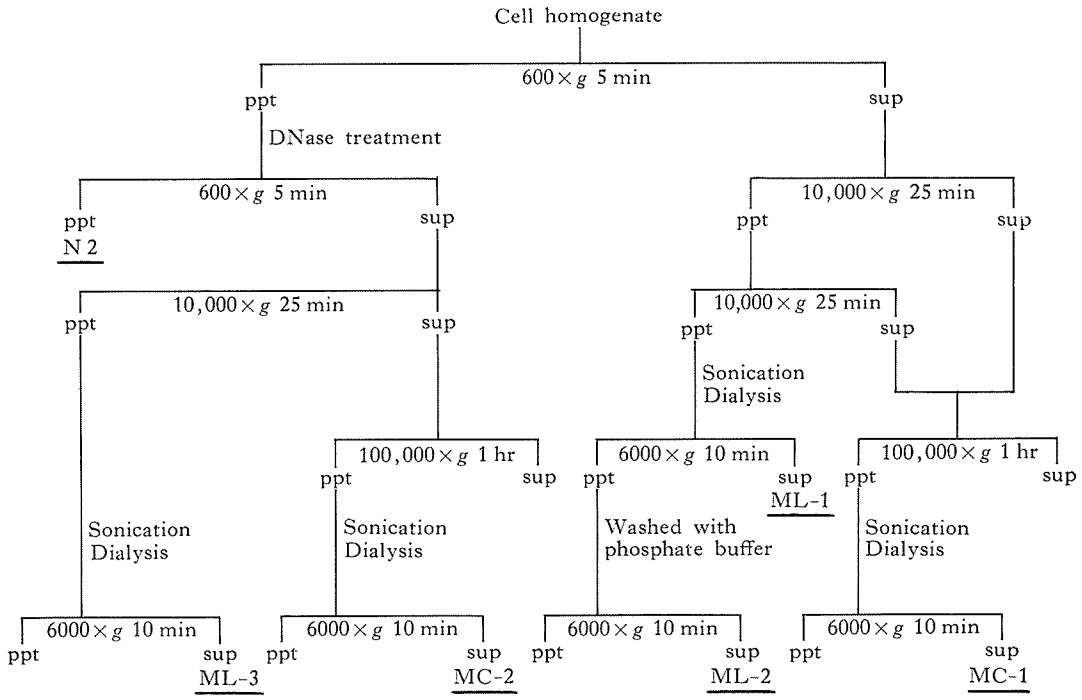

FIGURE 6 Procedures used for subcellular fractionation. The activity of each fraction is shown in Table 3.

TABLE 3 *Activity of subcellular fractions*

Fraction	Protein (mg/ml)	Final volume (ml)	Sample* (ml)	Activity ($-\triangle$OD)
N-2	11.0	10	0.3	0.13
			0.4	0.17
MC-1	2.40	15	0.3	0.13
			0.4	0.20
MC-2	1.50	10	0.4	0.20
			0.6	0.23
ML-1	0.22	15	2.0	0.04
ML-2	0.38	10	2.4	0.07
ML-3	0.25	10	2.4	0.03

* Sample volume used to estimate LEC activity

From these results, it seems that LEC activity is contained specifically in leucocytes.

6. *Stability of LEC activity*

With regard to the stability of the suspension, when suspended in 0.02 M phosphate buffer at pH 7.5 (original activity of 0.5 ml: $-\Delta$OD $=0.34$) LEC was quite labile and one half of the activity was lost after 60 min at 37°C or after 2 days at 0°C. This loss was attributable to the formation of a precipitate. When the suspension was heated at 56°C for 30 min, the activity was completely lost.

At below pH 6, LEC was precipitated as described in the previous report (AMANO *et al.*, 1956 a). When the pH of the suspension was adjusted to 11.5 and then centrifuged at 100,000 $\times g$ for 2 hours, the activity was found in the supernatant when it was neutralized. However, LEC activity was not solubilized by this treatment, because after recentrifugation of the neutralized supernatant at 100,000 $\times g$ for 2 hours, the activity was again detected in the precipitate.

It was found that the best way to obtain a stable suspension of LEC was to centrifuge it

and suspend the pellets in distilled water by sonication. The suspension in distilled water was quite stable, and 80 per cent of the activity (original activity of 0.5 ml: $-\Delta OD = 0.3$) was retained even after heating the preparation at 100°C for 15 min and at 0°C full activity could be restored for 10 days.

The effect of 8 M urea which is a protein denaturing agent was investigated. LEC was suspended in 8 M urea in distilled water and left overnight at 4°C and the suspension became less opalescent. After dialysis against distilled water, the opalescence increased to the same degree as that of the starting material suspended in distilled water and the activity was almost fully retained.

7. *Effects of hydrolytic enzymes*

The effects of the proteolytic enzymes trypsin and pronase were studied. When LEC was treated with 1 μg per ml final concentration of trypsin or pronase at 30°C for 1 hour, the activity was completely inactivated, as shown in Table 4. These results indicate that the particles bearing LEC activity consist of protein. However, neither DNA nor RNA have any relationship to the activity, because DNase and RNase had no effect on the activity. Consequently it is probable that the ribosomes contained in the LEC preparation do not contribute to its activity.

To see whether the active particles contain a lipoprotein layer(s) on their surface, the effect of " Habu " venom containing phosphatidase A was studied. As the native venom is known to contain several other enzymes and phosphatidase A is very heat stable, 10 mg per ml of venom solution in 0.05 M tris-buffer was heated at 100°C for 13 minutes. By this treatment proteolytic activity equivalent to 360 μg per ml of trypsin was completely inactivated and the activity of phosphatidase A was still retained. The heated venom solution was diluted 1: 300 to avoid damage of the bacterial cytoplasmic membrane. LEC activity was inactivated by 0.3 ml of the diluted venom, as shown in Table 5. The LEC inactivating and phosphatidase A activities of the heated " Habu " venom were inactivated by heat treatment at 100°C for 30 minutes at pH 11. These results suggest that the active particle has a membrane consisting of lipoprotein(s).

8. *Effects of enzyme inhibitors*

From the above results on the mechanism of

TABLE 4 *Inactivation of LEC activity by trypsin*

LEC (ml)	Trypsin (ml)	Soybean trypsin inhibitor (250 μg/ml) (ml)	Phosphate buffer (ml)	Distilled water (ml)		Activity ($-\triangle$OD)
0.5	0.2 (50 μg/ml)	—	0.3	0.5		0.03
0.5	0.2 (10 μg/ml)	—	0.3	0.5		0.03
0.5	0.2 (50 μg/ml)*	—	0.3	0.5		0.23
0.5	0.2 (10 μg/ml)*	—	0.3	0.5		0.23
0.5	0.2 (50 μg/ml)	0.2	0.1	0.5		0.23
0.5	0.2 (10 μg/ml)	0·2	0.1	0.5		0.22
0.5	—	—	0.5	0.5		0.24

Incubation 30°C 1 hr

* Heat inactivated trypsin

TABLE 5 *Inactivation of LEC activity by Habu venom*

LEC (ml)	Habu venom (ml)	Phosphate buffer (ml)	Distilled water (ml)		Activity (—△OD)
0.5	0.3 (× 300)	0.2	0.5		0.05
0.5	0.3 (× 900)	0.2	0.5		0.10
0.5	0.3 (×2700)	0.2	0.5	Incubation 35°C 1 hr	0.18
0.5	0.3 (×8100)	0.2	0.5		0.23
0.5	0.3 (× 300)*	0.2	0.5		0.22
0.5	0.3 (× 900)*	0.2	0.5		0.27
0.5	—	0.5	0.5		0.29

* Heat inactivated Habu venom at pH 11

the conversion of bacilli to spheroplasts, it is conceivable that some enzymic reaction(s) with LEC must proceed in advance of the second step of the reaction with lysozyme. To obtain evidence for an enzyme in the active particles of LEC, the effects of various enzyme inhibitors were investigated. The inhibitors used were, dicoumarol, ouabain, sodium azide, gramicidin S, Amytal, DNP, KCN, antimycin A and diisopropylfluorophosphate (DFP). The final concentrations tested were between M/1,000 and M/16,000 except with DFP, Amytal and gramicidin S. DFP was tested at final concentrations of between 0.02 M and M/1,000, gramicidin S at 100–0.1 μg per ml and Amytal at 10 mg–10 μg per ml. Among these, only dicoumarol was an effective inhibitor, as shown in Fig. 7, and at concentrations above M/8,000, LEC activity was completely inhibited and, in addition, the activity of lysozyme added for the assay was not affected by dicoumarol. Dicoumarol, like DNP and antimycin A, is known to inhibit the active uptake of divalent cations by mitochondria (ENGSTROM *et al.*, 1964). However, the behaviors of these uncouplers were unequivocal.

In the next experiment, dicoumarol was added to the assay system during the reaction of LEC and in this case it exhibited no inhibitory effect on LEC activity. These results indicate that dicoumarol inhibits the adsorption of active particles onto the bacterial surface.

9. *Activation of LEC activity by ATP and ADP*

Since dicoumarol is known to inhibit ATPase which plays the role of concentrating divalent cations and it inhibited LEC activity, as shown in the prior experiment, the effects of AMP, ADP, ATP and other triphosphates were examined. These nucleotides were dissolved in the M/15 phosphate buffer of the assay system

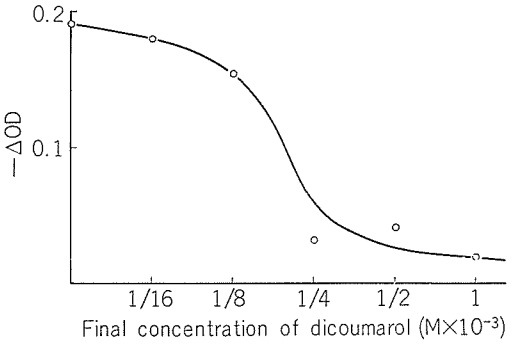

FIGURE 7 Inhibitory effect of dicoumarol.

to give the final concentrations required, so that each of the nucleotides was added together with LEC to the bacterial suspension in the sucrose medium. The results are shown in Table 6 and Fig. 8. ATP enhanced LEC activity and ADP did so also, but to a lesser degree. On the other hand, AMP had no activating effect. As the purity of the ADP used was more than 97 per cent, the effect of ADP cannot be regarded as due to contamination by ATP. Other triphosphates (GTP, UTP, CTP) were also found to exert the same enhancing effect, as shown in Table 6. In the control, the effect of ATP and lysozyme without LEC was examined. No spheroplasts were formed in the system.

Based upon these results, the ATPase activity of the LEC preparation was estimated according to the method of KIELLEY (1955). The assay system consisted of LEC (0.5 ml), 0.1 M tris-buffer (0.5 ml), 0.1 M KCl (0.2 ml), 0.01 M $MgCl_2$ (0.1 ml), H_2O (0.5 ml) and 0.01 M ATP (0.2 ml). After incubation, 2 ml of 5 per cent perchloric acid were added and the mixture was centrifuged. Inorganic P in the supernatant was determined by the method of Fiske-Subbarow. After 30 min incubation only 14 per cent of the added ATP had been hydrolyzed. Considering the amount of ATP used for activation of LEC activity, this result indicates that the activating effect of ATP on LEC activity cannot be explained by ATPase activity and that dicoumarol inhibited LEC activity in a different way from ATPase. In addition, ATP did not reactivate LEC which had been inhibited by dicoumarol.

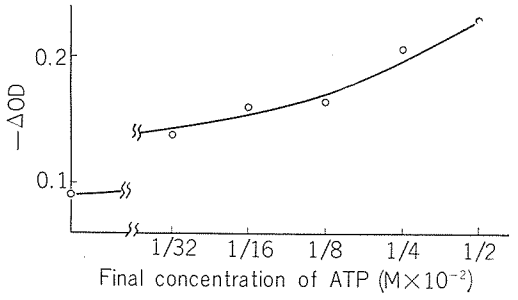

FIGURE 8 Activating effect of ATP.

TABLE 6 *Activating effect of ADP, ATP and other nucleoside triphosphates*

Nucleotide	Concentration	Activity (−△OD)
ATP	M/800	0.20
GTP	M/800	0.18
CTP	M/800	0.17
UTP	M/800	0.20
ADP	M/500	0.17
AMP	M/500	0.10
—	—	0.10

10. *Effects of metallic ions on LEC activity*

Since lysozyme was essential for spheroplasting by LEC, it was possible that LEC removed some metallic ions from the cell walls and so rendered the cell walls susceptible to lysozyme. If so, added metallic ions should inhibit LEC activity. To study this, varying concentrations of $CaCl_2$, $MgCl_2$, $CoSO_4$, $FeSO_4$ and $FeCl_3$ were respectively added to the assay system and LEC activity was estimated. In this experiment 0.05 M tris-buffer at pH 7.5 was used in place of M/15 phosphate buffer in the system shown in Table 1. As shown in Fig. 9, Fe^{3+} exerted the strongest inhibition and among the other ions tested the order of their inhibitory effects was $Cd^{2+} > Fe^{2+} > Ca^{2+} = Co^{2+} > Mg^{2+}$.

The preparation of LEC was preincubated with $CaCl_2$ in a final concentration of M/3,000 and then the mixture was added to assay system, in which the Ca^{2+} concentration was diluted to M/21,000. If the LEC preparation plus lysozyme was incubated with bacterial suspension in the presence of M/21,000 Ca^{2+} at the start, Ca^{2+} was not inhibitory. However, in this two step incubation experiment LEC activity was inhibited by Ca^{2+}, and ATP reactivated the inhibited LEC activity when it was added to the system at the second step (M/500 in final concentration). The same

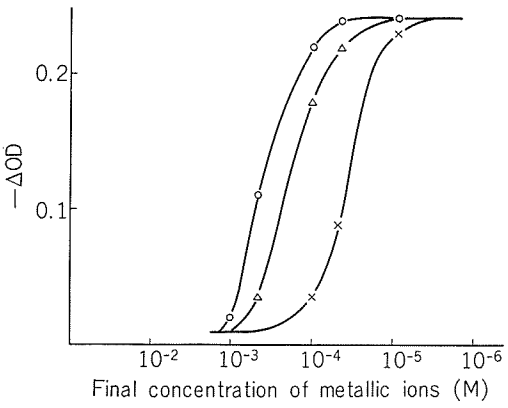

FIGURE 9 Inhibitory effects of metallic ions.
○———○ Mg^{2+} △———△ Ca^{2+}
×———× Fe^{3+}

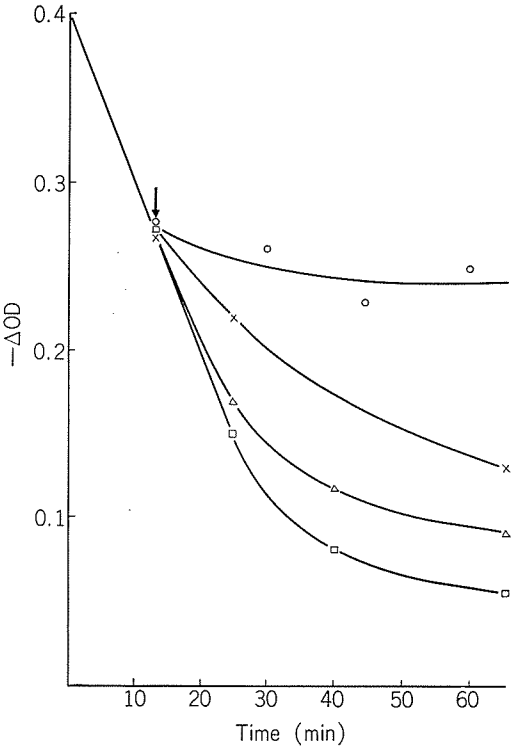

FIGURE 10 Effect of metallic ions during the reaction.
The arrow indicates the time at which the ions were added.
○———○ M/3,000 Ca^{2+}
×———× M/5,000 Fe^{3+}
△———△ M/10,000 Fe^{3+}
□———□ No ion added

phenomenon was observed with Mg^{2+}. In case of Fe^{3+}, ATP slightly reactivated LEC activity which had been inhibited by Fe^{3+}, as shown in Table 7. From these results it can be assumed that inhibitory metallic cations have some interaction with active particles of LEC and that Ca^{2+} and Mg^{2+} can be chelated by ATP, ADP and other tirphosphates resulting in reactivation of LEC.

In the next experiment, Fe^{3+} or Ca^{2+} was added to the assay system during the action of LEC and the effect was examined. The addition of Ca^{2+} (M/3,000 in final concentration) inhibited the activity of LEC immediately

TABLE 7 *Inactivation by preincubation with metallic ions and reactivation by ATP*

LEC (ml)	Tris buffer (ml)	$CaCl_2$ M/300 (ml)	$MgCl_2$ M/100 (ml)	$FeCl_3$ M/600 (ml)	Distilled water (ml)		ATP M/50 (ml)	Activity (−△OD)
0.5	0.2	0.1	—	—	0.2	Incubation, 37°C, 15 min	—	0.06
0.5	0.2	0.1	—	—	0.2		0.7	0.23
0.5	0.2	—	—	—	0.3		—	0.25
0.4	0.2	—	0.1	—	0.3		—	0.03
0.4	0.2	—	0.1	—	0.3		0.7	0.18
0.4	0.2	—	—	0.1	0.3		—	0.05
0.4	0.2	—	—	0.1	0.3		0.7	0.14
0.4	0.2	—	—	—	0.4		—	0.21

and no further increase in the number of spheroplasts was detected, as can be seen in Fig. 10. However, Fe^{3+} (M/5,000 or M/10,000 in final concentration), the most powerful inhibitor in the above experiment, did not inhibit the activity of LEC very much and the spheroplasting process, once started, proceeded even after the addition of Fe^{3+}, as also shown in Fig. 10. To see whether LEC particles were liberated from the bacterial surface by the addition of Ca^{2+} in this experiment, the following experiments were performed. The tubes were left for 15 min and when no further increase in spheroplasts was seen, the tubes were centrifuged. The supernatant was dialyzed and tested for LEC activity, but none was found. A similar experiment was performed with the cell walls of *E. coli* B. After incubation of LEC with cell walls, Ca^{2+} was added and the tube was again incubated for 15 min. Then the supernatants were centrifuged and dialyzed. No LEC activity was found in the dialyzate. In addition, even M/400 EDTA did not liberate LEC activity from the cell walls into the medium.

DISCUSSION

As shown in these experiments, the spheroplasting activity of leucozyme C can be distinguished as a two step reaction, 1) adsorption of active particles onto the surfaces of the sensitive organisms and concentration of divalent cations from the surfaces of the bacteria and 2) hydrolysis of mucopeptide by lysozyme contained in trace amounts in the leucozyme C preparation. The indispensability of lysozyme for the spheroplasting activity of leucozyme C was an unexpected finding, and this finding clarified the mechanism of the action of leucozyme C. However, it was difficult to elucidate the mechanism by which the action of LEC occurred before that of lysozyme.

The keys to this problem were the inhibition by dicoumarol and by metallic cations and the activation by ADP, ATP and other nucleoside triphosphates. From these findings it was suspected that the active concentration of metallic cations by LEC particles was essential for LEC activity. However, the very weak ATPase activity detected in the LEC preparation disproved this possibility. The chief findings, which led us to assume that the binding of metallic cations is essential for LEC activity, were as follows: 1) LEC activity was inhibited by preincubation with a certain concentration of Ca^{2+}, 2) the same amount of Ca^{2+} did not inhibit the activity when directly added to the assay system and not preincubated with it, and 3) when LEC activity was inhibited by preincubation with Ca^{2+} it could be reactivated by addition of ATP. These findings and the result of kinetic studies on LEC activity led us to assume that the multiply adsorbed LEC particles chelate Ca^{2+} and Mg^{2+} from the bacterial surface and hence render the cell walls lysozyme sensitive.

The behavior of ferric ions was found to be somewhat different from that of Ca^{2+} ions. Thus Fe^{3+} did not inhibit the spheroplasting process when once started and inhibit its initiation. These results can be explained by assuming that Fe^{3+} inhibited the adsorption process. For the same reason, it can be assumed that dicoumarol also inhibited the adsorption process.

The stability of the LEC preparation on boiling at 100°C for 15 min and its resistance to 8 M urea were unexpected results and forced us to reject the idea that the LEC activity was enzymatic. The LEC activity can be understood as a simple physicochemical reaction, namely chelation.

The present studies also demonstrated that the LEC activity was localized in the microsomal fraction of the leucocytes. The microsomes are derived from the cell membranes (including nuclear membranes) and endoplasmic reticula. However, endoplasmic reticula are not well developed in leucocytes. From this reason, the microsomes of leucocytes can be assumed to be derived mainly from cell membranes and nuclear membranes. In leucocytes, phagocytic vacuoles are formed by

invagination of the outer cell membranes (GOODMAN, 1956), and hence it is probable that the membranes lining the phagocytic vacuoles also have the ability to chelate metallic ions, and this renders phagocytosed Gram-negative bacteria lysozyme sensitive. Lysozyme can easily be obtained in the vacuole fluid by disruption of the granules (ZUCKER-FRANKLIN *et al.*, 1964) and also from the cytoplasmic fluid.

MIYAMA *et al.* (1959) have reported that if purified colicine K-O antigen was incubated with a crude preparation of leucozyme C the antibiotic activity of colicine K was inactivated by boiling for 10 min whereas colicine K-O antigen itself was quite stable. They postulated that the antibiotic moiety (colicine K protein) was separated from the colicine K-O antigen complex by the action of the leucozyme C. These results were reconfirmed using an LEC preparation. From the present studies, the previous results may be understood as follows; LEC interacted with or bound colicine K-O antigen and changed the binding state of colicine K-protein causing the colicine K-protein to become heat labile. In addition, the experiment was conducted in buffered saline, and in this buffer LEC particles would have been aggregated on boiling.

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REFERENCES

AMANO, T., SEKI, Y., FUJIKAWA, K., KASHIBA, S., MORIOKA, T. and ICHIKAWA, S. (1956 a). The isolation and characterization of protoplasts from *Escherichia coil* B with the treatment of leucocytes extract. *Med. J. Osaka Univ.* **7**, 245–256.

AMANO, T., SEKI, Y., KASHIBA, S., FUJIKAWA, K., MORIOKA, T. and ICHIKAWA, S. (1956 b). The effects of leucocytes extract on *Staphylococcus pyogenes*. *Med. J. Osaka Univ.* **7**, 233–243.

BROWN, D. D. and KOZLOFF, L. M. (1957). Morphological localization of the bacteriophage tail enzyme. *J. Biol. Chem.* **225**, 1–11.

COHN, Z. A. and HIRSCH, J. G. (1960). The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leucocytes. *J. Exptl. Med.* **112**, 983–1004.

ENGSTROM, G. W. and DELUCA, H. F. (1964). The nature of Ca^{2+} binding by kidney mitochondria. *Biochemistry* **3**, 379–383.

GOODMAN, J. G. and MOORE, R. E. (1956). Electron microscopic study of phagocytosis of staphylococcus by human leucocytes. *J. Bacteriol.* **71**, 547–556.

KIELLEY, W. W. (1955). [95] Mg-activated muscle ATPase. In the *Methods in Enzymology* Vol. II. Academic Press Inc., New York. 588–591.

MANDELSTAM, J. (1961). Isolation of lysozyme soluble mucopeptides from the cell wall of *Escherichia coli*. *Nature* **189**, 855–856.

MIYAMA, A., ICHIKAWA, S. and AMANO, T. (1959). Spheroplasts of colicin K producing *Escherichia coli* K235 prepared by leucozyme C and by lysozyme. *Biken's J.* **2**, 177–185.

WARREN, L. (1959). The thiobarbituric acid assay of sialic acid. *J. Biol. Chem.* **334**, 1971–1975.

WEIDEL, W., FRANK, H. and MARTIN, H. H. (1960). The rigid layer of the cell wall of *Escherichia coli* strain B. *J. Gen. Microbiol.* **22**, 158–166.

ZUCKER-FRANKLIN, D. and HIRSCH, J. G. (1964). Electron microscope studies on the degranulation of rabbit peritoneal leucocytes during phagocytosis. *J. Exptl. Med.* **120**, 569–576.