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Decapsulation of *Bacillus megaterium**

MITSUO TORII, OSAMU KURIMURA, SAYAKA UTSUMI, HARUO NOZU AND TSUNEHISA AMANO

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

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SUMMARY

The decapsulating agent of the dog liver extract active against living encapsulated *B. megaterium* was proved not to be tissue lysozyme. This is contrary to the results of the lysozyme-mediated decapsulation of the formalin-killed organisms reported by Welshimer (1953). The decapsulated cells were still alive and capable of being encapsulated when grown on a new medium.

The released capsular substances were separated into three fractions by electrophoresis. One was proved to be pure polyglutamate and other two were the association products of polyglutamate with other substances such as antigenic polysaccharide. These results indicate the complex structure of the capsules of *B. megaterium*.

INTRODUCTION

Enzymatic destruction of the bacterial capsules in several instances has been reported. Dubos and Avery (1931) demonstrated an enzyme from a soil organism capable of hydrolyzing type III pneumococcal capsular polysaccharide, not only when purified, but also in the capsular condition with the intact cell. McClean (1941) described hyaluronidase, which decomposed hyaluronic acid capsules of *Streptococcus* group A and C. Humphries (1948) found a phage enzyme which splits the capsules of *Klebsiella aerogenes*.

The decapsulation of *B. anthracis* was first described by Cromartie *et al.* (1947) in their histological studies of the infected foci of resistant animals and immunized rabbits. Nordberg (1955) found in *in vitro* experiment that egg white lysozyme was effective in destroying the capsules of *B. anthracis*. Torii (1955), one of the present authors, also found an enzyme-like agent in dog liver extracts capable of splitting the capsules of the same organism. Welshimer (1953) described lysozyme-mediated decapsulation of formalin-killed *B. megaterium* and Tomcsik and Guex-Holzer (1952) reported lysozyme mediated decapsulation of living *Bacillus M*. As Torii (1955) also reported that his dog liver extract could destroy the capsules of *B. megaterium*, the authors restudied the nature of the decapsulating agent for encapsulated *B. megaterium* and *B. anthracis*, to see whether it was tissue lysozyme, or a quite distinct entity.

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The present paper reports separation of the decapsulating agent from tissue lysozyme and studies on the chemical nature of the capsular substances liberated from encapsulated *B. megaterium* during decapsulation by a partially purified preparation.

MATERIALS AND METHODS

1. *Bacteria*

Bacillus megaterium strain A5: The strain was kindly sent by Prof. J. Tomcsik from the University of Basel. The name was given to the strain in this laboratory.

Bacillus anthracis strain Vollum: This strain was also furnished through the courtesy of Prof. J. Tomcsik.

2. *Culture medium*

B. megaterium: A small population of *B. megaterium* was seeded in a medium containing Polypeptone (1%, Takeda Co.), casein hydrolyzate (0.2%), NaCl (0.2%), KH_2PO_4 (0.3%), yeast extract (0.5%) at pH 7.0. The medium was incubated with mechanical shaking.

B. anthracis: To 89 ml of meat infusion were added 1.0 g of Proteose peptone (Difco), and 0.5 g of NaCl. The pH was adjusted to 6.8. To the sterilyzed solution were added 11 ml of sterile 7 per cent $NaHCO_3$. The medium was seeded and the container stoppered and incubated with mechanical shaking.

3. Antisera

Polyglutamate antiserum: The saline suspension of heat killed encapsulated cells of *B. anthracis* was repeatedly injected intravenously into rabbits according to the description of Tomcsik and Ivánovics (1938).

B. megaterium antiserum: The saline suspension of heat killed encapsulated cells of *B. megaterium* was repeatedly injected intravenously into rabbits. After bleeding the animals, the antiserum from each rabbit was tested for the precipitin reaction with capsular polysacharide and with the capsular polyglutamate of *B. megaterium*.. The antisera, which gave a positive precipitin reaction with polysaccharide and not with polyglutamate, were selectively used in this experiment. Samples of polysaccharide and polyglutamate of *B. megaterium* used as antigens were the same samples as described in our previous paper (Utsumi *et al.*, 1959).

4. *Detection of decapsulation*

To 1.0 ml of saline suspension of the encapsulated organisms was added 0.1 (or 0.2) ml of the enzyme preparation and the mixture was incubated at 37°C. After from 10 to 30 min. incubation a loopful was examined under a microscope by the indian ink method.

5. *Quantitative determination of polysaccharide*

The anthrone method was employed (Chung *et al.*, 1954).

6. *Quantitative determination of polyglutamate*

Kream's safranin method was used (1954). To 3.0 ml of the reaction mixture were added 7.0 ml of 20 per cent trichloroacetic acid solution and the centrifuged supernatant was shaken three times with 3.0 ml aliquots of ether to remove trichloroacetic acid. 2.0 ml of the water layer were put in a 10 ml volumetric flask containing 2.0 ml of 0.1 per cent safranin 0 and 2.0 ml of citric acid buffer at pH 5.9. The volume was brought up to 10.0 ml by adding saline. After standing for 30 min. at room temperature, the insoluble complex of

polyglutamate and safranin 0 was centrifuged down and 0.4 ml of the supernatant was diluted to 10.0 ml. The optical density of remaining safranin 0 in the diluted supernatant was estimated at 520 mμ by a Coleman junior spectrophotometer using a model 6–310 B cuvette. The amount of polyglutamate was calculated through the amount of safranin 0 precipitated.

7. *Detection of polyglutamic acid on paper strips after electrophoresis*

The details of the method are described in another paper in this journal (Torii, 1959c).

RESULTS

1. *Separation of the decapsulating agent from lysozyme in a dog liver extract*

For this study it was tried to remove a lysozyme activity from the crude extract of a dog liver.

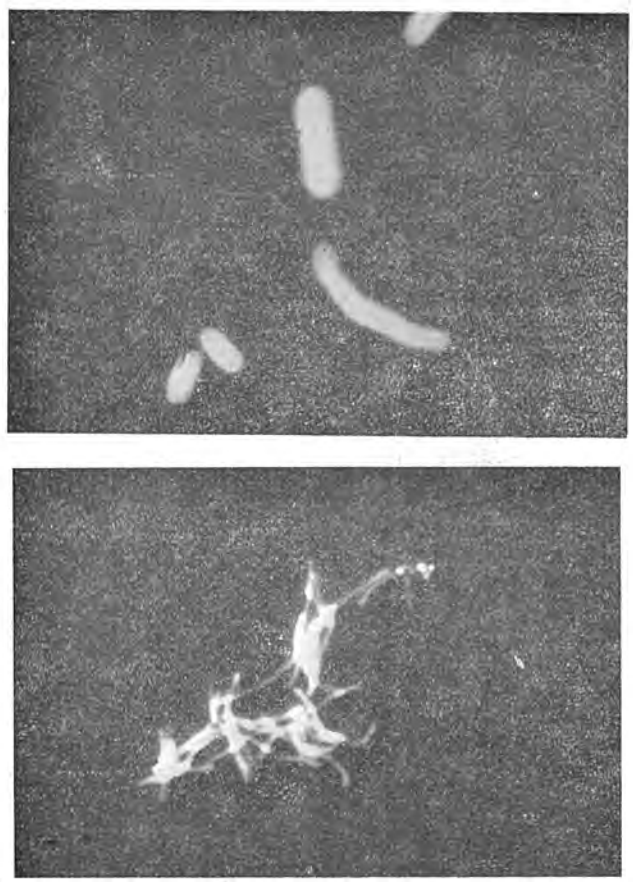

Fig. 1. Action of the decapsulating agent toward encapsulated *B. megaterium*.
A: Encapsulated *B. megaterium*
B: *B. megaterium* decapsulated by the decapsulating agen.

A fresh dog liver was homogenized with twice its weight of saline containing 0.1 N acetic acid, and freezing and thawing was repeated several times. After centrifugation, the supernatant was neutralyzed to pH 7.0 and again centrifuged. The supernatant was fractionated with ammonium sulfate and the precipitate obtained between 66 and 100 per cent saturation was dissolved in a minimal amount of water and dialyzed against saline. Bentonite (Wako Pure Chemicals) was added to the dialyzed solution (crude extract) at a rate of 15 mg per ml and the mixture stirred well for 5 min. at room temperature. The pH of the red supernatant after centrifigation (R-fraction) was adjusted to 3.0, then immediately neutralized and centrifuged. A yellowish supernatant was obtained (Y-fraction).

Y and R fractions were very active in destroying the capsules of living *B. megaterium*. The capsules disappeared completely within 5 minutes as shown in Fig. 1. During decapsulation, the chains of the bacilli were not separated into individual cells.

When a crude extract was added to a suspension of living encapsulated *B. megaterium*, decapsulation and bacteriolysis proceeded in parallel and after from 5 to 15 min. the turbid suspension became almost clear. The crude extract contained 30 μg per ml of lysozyme, estimated as egg white lysozyme. The bacteriolytic activity of lysozyme could be completely inhibited by 0.1 M magnesium sulfate in final concentration. The decapsulating activity was still restored in the presence of Mg^{++}. The effect of egg white lysozyme (0.5—10 μg) was examined in the presence of Mg^{++}. Lysozyme in the presence of Mg^{++} could not destroy the capsules. From these experiments it can be stated that lysozyme plays no role in the decapsulation, the decapsulating agent being an entirely distinct entity.

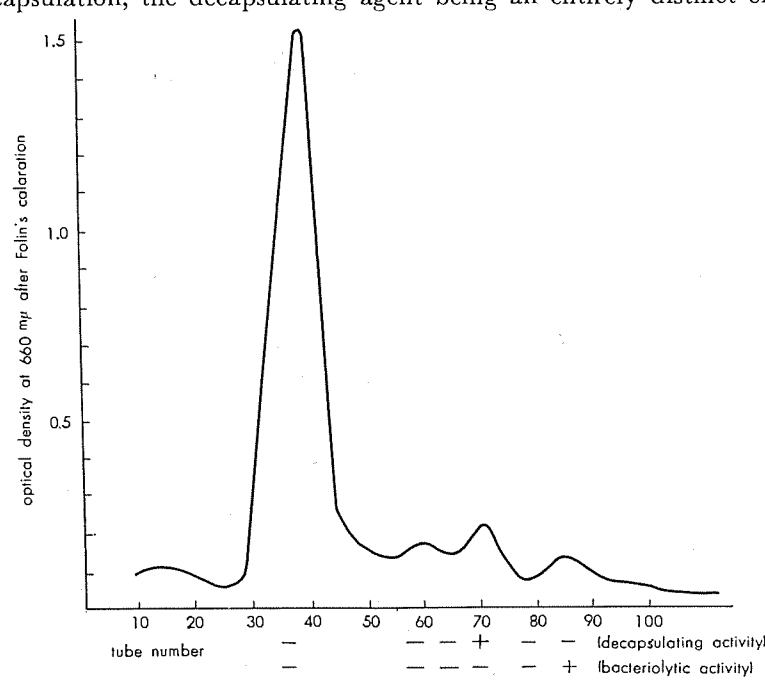

Fig. 2. Chromatographic diagram of crude dog liver extract.
Column, 3 × 30 cm of bentonite-hyflo supercel mixture (1 : 10); gradient elution, from saline to sulfuric acid in 20 per cent pyridine (pH 4.0); 5ml per tube.

This was confirmed by column chromatography using bentonite as an adsorbent. A glass cylinder (3 × 40 cm) was filled with a mixture of bentonite and Hyflo supercel (1:10) to a height of 30 cm. 10 ml of the crude extract was added to the top of the column. Gradient elution was carried out from saline to 20 per cent pyridine-sulfuric acid (pH 4.0). Each fraction was dialyzed against saline and then assayed for its decapsulating and bacteriolytic activity. The results are shown in Fig. 2.

As can be seen from the figure, the decapsulating and bacteriolytic activities were separated. Thus the decapsulating agent and tissue lysozyme are definitely quite distinct entities.

2. *Effect of pH and heat stability of the decapsulating activity*

The Y fraction was used in this test. As shown in Fig. 3 the activity was expressed by the highest dilution which permitted splitting of the capsules in 20 min. incubation. The highest activity was found at pH 3.6 to 4.5. Though the control cells were not agglutinated at a pH lower than 5.0, the decapsulated cells were agglutinated, as shown in Fig. 1 B. Following experiments were performed at about pH 6.0 unless otherwise mentioned.

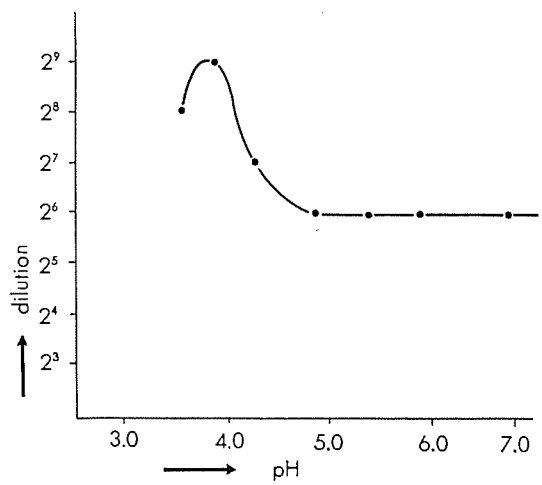

Fig. 3. Effect of pH on the decapsulating activity.

The heat stability was examined at pH 3.0, 7.0 and 8.0. At these test pHs, the activity was lost by heating at 56°C for 30 min.

3. *Fate of decapsulated cells of B. megaterium*

Dubos and Avery (1931) stated that type III pneumococci, when decapsulated by the enzyme splitting type III polysaccharide, could grow and in addition the bacteria were able to reproduce their capsules on further incubation. After the capsules of *B. megaterium* had been destroyed by treatment with the Y fraction, the fate of the bacteria was investigated.

To 2.0 ml of the encapsulated bacterial suspension containing 3.5×10^7 cells (chains) per ml were added 0.4 ml of the Y fraction. A tube containing the same amount of bacterial suspension and 0.4 ml of saline served as a control. After the tubes had been incubated at 37°C for 10 min. and complete decapsulation had been observed, viable counts were made.

Table 1. Viable counts of decapsulated cells and normal cells

	Test	Control
Bacterial suspension	2.0 ml	2.0 ml
Liver extract	0.4	—
Saline	—	0.4
Viable cells	3.2×10^7	3.5×10^7

As shown in Table 1, the decapsulated cells were still alive. When such decapsulated cells were grown on the surface of nutrient agar, all the colonies were mucoid and the cells were again encapsulated. It is very interesting that the decapsulated cells of *B. megaterium* behave in the same manner as the cells of type III pneumococci when they grown in a fresh medium.

4. *Substances liberated from the capsules of B. megaterium*

It is well-known that the capsules of *B. megaterium* are composed of polysaccharide and polyglutamate, and the chemical structure of the latter has been shown in this laboratory to be γ-copolymers of D- and L-glutamic acid (Torii, 1959a; Utsumi *et al.*, 1959). To confirm the liberation of the capsular materials during decapsulation, polyglutamate and polysaccharide liberated from the capsules by the R fraction were estimated.

The encapsulated cells were washed three times with saline and resuspended in saline. To the dense suspension was added 1/10 its volume of the R fraction. The mixture was incubated at 37°C. At the time intervals shown in Fig. 4, samples were taken and the amounts of polyglutamate and polysaccharide were estimated.

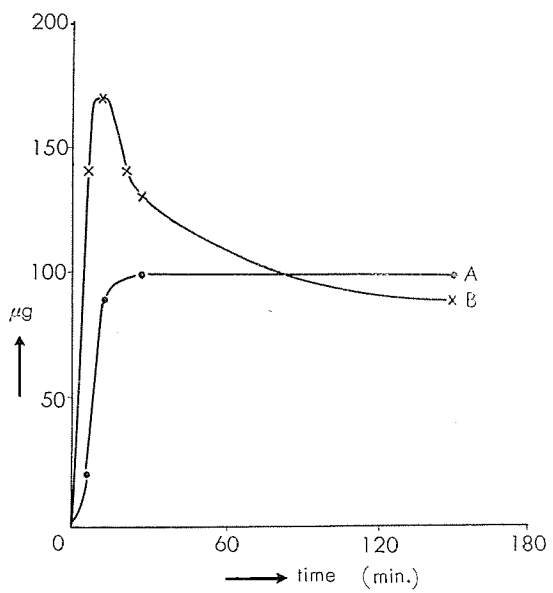

Fig. 4. Capsular substances released from encapsulated *B. megaterium* by the decapsulating agent.
A: Polysaccharide (by anthrone method)
B: Polyglutamate (by safranin method)

As can be seen in Fig. 4, polysaccharide and polyglutamate were liberated during decapsulation. When decapsulation was complete, the amounts of polyglutamate and polysaccharide reached maxima. The curve of polysaccharide concentration showed a plateau thereafter, but that of polyglutamate declined after decapsulation was complete. The disappearance of polyglutamate was partly due to the hydrolysis by γ-glutamylase (Torii, 1959b) and the small peptides thus formed could not be precipitated by safranin O.

5. *Precipitin reaction of polyglutamate and polysaccharide liberated from the capsules*

As the polysaccharide was estimated by the anthrone method, the presence of anthrone positive substances does not necessarily imply the presence of polysaccharide. To prove the presence of polysaccharide in the medium the precipitin reaction was performed and the same reaction was also carried out for polyglutamate.

To 30 ml of a dense suspension of the encapsulated cells was added 1.3 ml of the Y fraction. The mixture was incubated at 37°C for 30 min. when complete decapsulation was confirmed. To the supernatant after centrifugation was added trichloroacetic acid at a concentration of 2 per cent to remove protein. The mixture was centrifuged. The supernatant was shaken four times with ether, neutralized and finally lyophilized. The lyophilized material was used as antigen for the qualitative precipitin reaction. As control antigens, polyglutamate and polysaccharide of *B. mega*erium were used. Polyglutamate was prepared from the encapsulated cells according to the description of Hanby and Rydon (1946) and a solution was made by dissolving 1.0 mg in 10 ml of saline. Polysaccharide was extracted from the encapsulated cells according to the method of Tomcsik and Guex-Holzer (1951). The antigen solution used was prepared by dissolving 1 mg in 10 ml of saline.

As shown in Table 2, polyglutamate and polysaccharide were shown to be liberated from the capsules.

Table 2. Precipitin reaction of capsular substances released from *B. megaterium* by the dog liver extract

Antigens / Antisera	Polyglutamate	Capsular polysaccharide	Capsular substances released from *B. megaterium* by liver extract
Polyglutamate antiserum	+	−	+
Polysaccharide antiserum	−	+	+
Normal serum	−	−	−

6. *Electrophoretic fractionation of capsular substances liberated by the decapsulating agent*

This experiment was made to see, whether polyglutamate and capsular polysaccharide were associated.

The preparation of the capsular substances was the same as that in the prior experiment except that the supernatant (Tube No. 1) containing the capsular substances separated from the bacterial cells after 30 min. incubation was divided into two equal portions. One portion was further incubated at 37°C for 4 hours and then deproteinized as described above. The other portion was immediately deproteinized. The tube (No. 2) containing the bacterial suspension and saline in place of the Y fraction and the tube (No. 3) containing

the Y fraction and saline in place of bacterial suspension served as controls. They were treated in the same way as the experimental tubes. Each batch of lyophilized materials was dissolved in 0.2 ml of 1/15 M sodium acetate and streaked on filter paper (Toyo filter paper No. 51, 2 × 40 cm). Electrophoresis was carried out at 500 V for 2 hours in 1/15 M sodium acetate. After electrophoresis, the paper strips were dried and polyglutamate was detected by the technique described by Torii (1959c). The results are shown in Fig. 5.

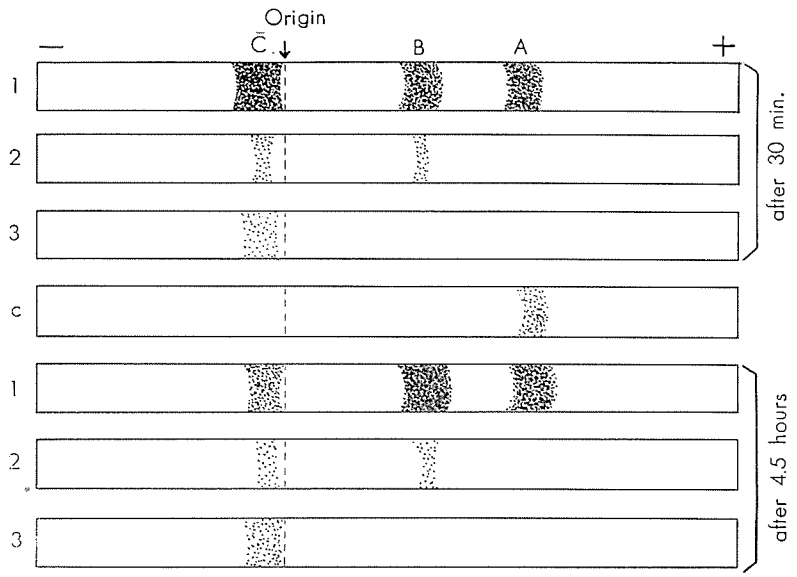

Fig. 5. Paper electrophoretic diagrams of capsular substances released from encapsulated *B. megaterium* by the decapsulating agent.
1. Encapsulated cells + decapsulating agent
2. Encapsulated cells + saline
3. Saline + decapsulating agent
C. Polyglutamate

As can be seen from the figure, three spots of polyglutamate were found. Spot A corresponded to an authentic sample of polyglutamate. Each spot of the sample after 30 min. incubation was compared with the corresponding spot of the sample after 4.5 hours incubation. Spot C of the latter was far fainter and spots A and B of the latter were much stronger than those of the former.

The same experiment was repeated on a larger scale. Four paper strips were used. One was used for the detection of three spots of polyglutamate. The part of each of the three strips corresponding to each spot was cut and soaked in 5.0 ml of water at room temperature for 10 min. The other parts of the three strips not corresponding to the

Table 3. Precipitin reaction of the fractions shown in Fig. 5

Antisera \ Fraction	A	B	C
Polyglutamate antiserum	+	++	—
Polysaccharide antiserum	—	—	+

spots were also soaked in the same way. Each extract was lyophilized and the residue was dissolved in 0.5 ml of saline and used as antigen for the precipitin reaction.

As can be seen in Table 3, spots A and B contained antigenic polyglutamate and spot C antigenic polysaccharide. The extracts from the pieces not corresponding to spots did not contain antigenic substances. It is very peculiar that the antigen of spot C did not precipitate with polyglutamate antiserum, although spot C gave a positive polyglutamate reaction on paper.

The following experiment was performed to prove that spot C really contained polyglutamate. Spots A and B were also tested.

The same experiment was repeated in a larger scale. The parts at 6 paper strips corresponding to each spot of polyglutamate were heated at 100°C in a tube containing 5.0 ml of water. The extract was hydrolyzed with 6 N hydrochloric acid for 6 hours at 100°C, concentrated and finally dried *in vacuo*. The residues were dissolved in 0.2 ml of 1/15 M sodium acetate and 0.02 ml of the solution was streaked on paper. Electrophoresis was carried out under the condition described above. After drying the strips they were cut in two longitudinally. One piece was used for the detection of ninhydrin positive substances. The other was used for the detection of reducing sugars with ammoniacal silver nitrate. The results are presented in Fig. 6.

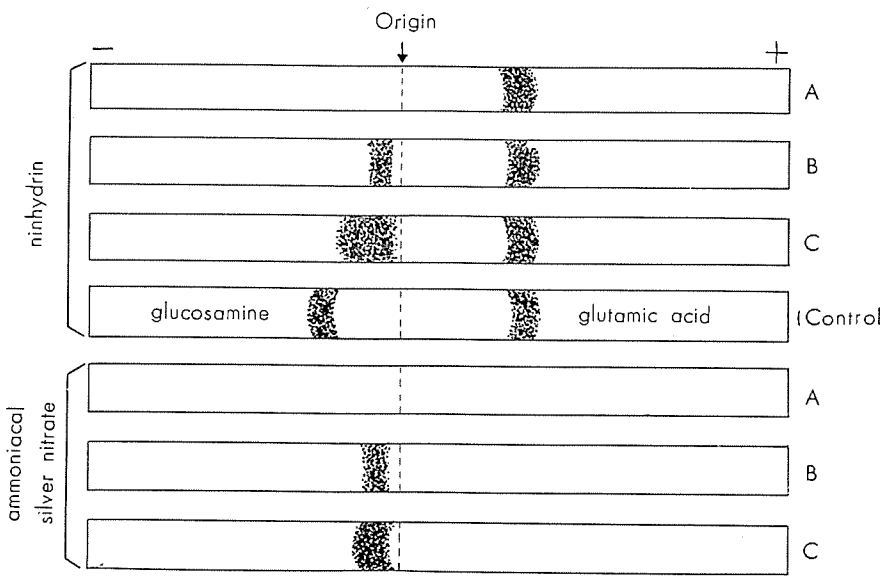

Fig. 6. Paper electrophoretic diagrams of the hydrolyzates of fractions shown in Fig. 5.
A: hydrolyzate of A shown in Fig. 5
B: hydrolyzate of B shown in Fig. 5
C: hydrolyzate of C shown in Fig. 5

As shown in the figure, after hydrolysis of spot A only glutamic acid was detected. Thus it is obvious that the substance in spot A was polyglutamate.

From both spot B and C considerable amounts of glutamic acid were detected and additional another ninhydrin positive substance was found in both hydrolyzates. The location of the second ninhydrin positive substance was superimposed

on the brown spot given by ammoniacal silver nitrate. The electrophoretic mobility to the cathode of this second fraction at pH 7.2 was the same as that of DNP-ethanolamine and of monoamino-monocarboxylic acid, and it was less than that of glucosamine. From these results it is obvious that the second fraction contained some reducing suger(s) and ninhydrin positive substance(s) other than glutamic acid.

On consideration of the results of the precipitin reaction and of electrophoretic analysis on the hydrolyzates, it is concluded that spot B was a polyglutamate associated with a nonantigenic substance(s) other than polyglutamate and that spot C was an unidentified antigenic polysaccharide together with unknown ninhydrin positive substances and polyglutamic acid. Though the presence of polyglutamic acid in spot C was not proved by the precipitin reaction, its presence can be assumed from the fact that spot C decreased while spots A and B increased when the supernatant containing the capsular material and decapsulating agent was incubated for a further 4.0 hours.

From these results it can be stated that the capsules of *B. megaterium* are composed of polyglutamic acid together with an antigenic polysaccharide and an unidentified substance(s).

DISCUSSION

There are at present two methods to study the structure of bacterial capsules. The one is the chemical extraction and purification in combination with the capsular reaction, by which Tomcsik succeeded in clarifying the structure of the capsules of organisms of genus *Bacillus*. The other is to investigate the course of the destruction of capsules by certain enzymes. This method has been adopted by Dubos and Avery (1931) in their studies of the capsules of type III pneumococcus. Torii (1955) made use of the second method to study the capsular structure of *B. megaterium* and *B. anthracis*. He employed a dog liver extract which could decapsulate *B. anthracis* and *B. megaterium*. In addition, he demonstrated the hydrolysis of the capsular polyglutamate of the latter but not of the former organisms. Welshimer (1953) showed that egg-white lysozyme destroyed the capsules of formaline-killed *B. megaterium*. The crude dog liver extract Torii used was later shown to contain tissue lysozyme. As described in the present report, lysozyme could be separated by chemical fractionation from the decapsulating agent. Thus the role of lysozyme in the processes of the decapsulation of living *B. megaterium* by the dog liver extract was excluded.

Later Torii (1959b) proved that the hydrolysis of polyglutamate of *B. megaterium* was due to the enzyme, "γ-glutamylase", chemical fractionation of which had not achieved a separation from the decapsulating agent. γ-Glutamylase could not hydrolyse D-γ-glutamyl polypeptide of *B. anthracis*. The acid hydrolyzate of polyglutamate of *B. anthracis* contains a very small amount of L-glutamate, the amount of which may be explained by a partial racemization during hydrolysis (Torii 1956). If the polyglutamate of *B. anthracis* really contains L-glutamate, it seems probable that the decapsulation of *B. anthracis* and *B. megaterium* was caused by γ-glutamylase. However at present the nature of the decapsulating agent is

still obscure. Enzymes like trypsin, pepsin, papain, *B. subtilis* protease, Taka-diastase, amylase and hyaluronidase do not decapsulate *B. megaterium*. The active agent of the dog liver extract cannot be inhibited by diisopropyl-fluorophosphate. It is inactivated by heating at 56° C for 30 min. and also by proteolytic enzymes. It is ineffective against the capsules of some *pneumococci*, some *Klebsiella pneumoniae*, *Leuconostoc dextranicum* and some *E. coli*. Future work on the purification of γ-glutamylase will solve the problem.

The capsular substances released during decapsulation were not homogeneous as shown by electrophoresis. They were separated into three fractions. The component moving rapidly to the anode (spot A) was pure polyglutamate. The other two components (spots B and C) contain other substances in addition to polyglutamate. The presence of polyglutamate in spot C was indicated by the fact that after a prolonged incubation of 4.5 hours spot C became very faint and spot A and B stronger. It cannot yet be explained why the polyglutamate of spot C was not precipitated by its antiserum and why spot C gave a positive polyglutamate reaction. Though the chemical reaction used for the detection of polyglutamate on paper is not perfectly specific for polyglutamate (Torii, 1959c), probably polyglutamate is associated with other substances like antigenic polysaccharide and is covered by the substances so that it cannot move on electrophoresis at pH 7.2 and cannot combine with polyglutamate antiserum. The polyglutamate in spot B is likewise associated with other components and consequently electrophoretic mobility to the anode was less than that of pure polyglutamate (spot A).

Attempts to purify the decapsulating agent are still continuing in this laboratory. The more detailed structure of the capsules of *B. megaterium* will be reported after pure decapsulating agent will be obtained.

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