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IDENTITY OF MEGACIN A WITH PHOSPHOLIPASE A

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SUMMARY Synthesis of megacin A, a bacteriocin of *Bacillus megaterium* strain 216, was induced by N-methyl-N′-nitro-N-nitrosoguanidine. Megacin A was highly purified and this purified preparation had demonstrable phospholipase A activity. Both activities were compared at each purification step, and were found to be purified in parallel. The sedimentation rates upon ultracentrifugation and the electrophoretic mobilities of the two activities were the same and both activities were inactivated at the same rate by treatment at 100°C. Megacin A negative mutants were all deficient in phospholipase A. These facts suggested that megacin A and the phospholipase A activity are two aspects of the same molecule.

Immunochemical studies showed clearly that both active sites resided in a single molecule.

All the strains of *Bacillus anthracis* tested were sensitive to megacin A in medium containing $NaHCO_3$.

INTRODUCTION

A bacteriocin of *Bacillus megaterium*, megacin A, was first described by IVÁNOVICS and ALFÖLDI (1954) and studied further by IVANOVICS and his colleagues (1955 a, 1955 b, 1957 a, 1957 b, 1958, 1959 a and 1959 b). According to their results, 1) megacin A is produced by the megacinogenic strain 216 following UV or mitomycin C in duction (Marjai, 1964), without producing phage; and it is lethal for cells of most *B. megaterium* strains, including the producer (strain 216), though to a lesser degree than to the others; 2) among the various bacterial strains belonging to the genus *Bacillus* tested, all the *B. megaterium* strains, 8 of 43 strains of *B. anthracis*, 2 of 13 strains of *B. subtilis* were sensitive, while all the *B. cereus* strains were insensitive, despite their biological similarity; 3) megacin A is moderately thermolabile, being precipitated at 75 per cent saturation with ammonium sulfate, and is nondialyzable; 4) it is antigenic, for rabbit antisera can precipitate megacin and neutralize its bactericidal activity; 5) it kills sensitive microorganisms evoking cytoplasmic lysis; 6) the protoplasts of sensitive strains are lysed by megacin A in a sucrose medium of high osmotic pressure.

HOLLAND (1961, 1962) obtained a highly purified preparation of megacin A but failed in clarifying its enzymic nature.

IVÁNOVICS, ALFÖLDI and NAGY (1959) described the lysis of protoplasts of sensitive

strains by megacin A, and they discussed the similarity and dissimilarity of megacin A to plakin, which was hypothesized to be a kind of phospholipase (HIGASHI *et al.*, 1963). The studies on the nature of plakin have been continued and very recently evidence has been presented which suggests that it is phospholipase A (HIGASHI *et al.*, 1966).

Since IVÁNOVICS kindly enabled us to study megacin A by giving us the megacinogenic strain 216, comparative studies of megacin A and plakin have been continued to test the speculation that megacin A may be phospholipase A. The present paper describes the results of these studies.

MATERIALS AND METHODS

1. *Bacterial strains used*

Bacillus megaterium strain 216, producing both megacin A and C, was used as a sources of megacin A. Strain KM of *B. megaterium* was most sensitive to megacin A and was used as indicator. To test the sensitivity of *B. anthracis* to megacin A, strains Vollum, Kyoto, Wadayama, NP, Stern and HM were used.

2. *Media*

For production of megacin A, medium I was used. It consisted of polypeptone (Daigo Eiyo Co., Japan) 10 g, meat extract (Mikuni Kagaku Co., Japan) 5 g, yeast extract (Daigo Eiyo) 5 g, NaCl 5 g, H_2O 1000 ml (pH=7.2).

Nutrient agar plates were used for the titration of megacin. They consisted of polypeptone 10 g, meat extract 10 g, NaCl 3 g, Agar (Wako Pure Chemicals Ltd., Japan) 15 g, H_2O 1000 ml.

3. *Megacin assay*

Titration of megacin activity was the same as described by IVÁNOVICS *et al.* (1957) except that *B. megaterium* strain KM was used as an indicator.

4. *Protoplasts*

Preparation of protoplasts of *B. megaterium* strain KM was as described by HIGASHI *et al.* (1963).

5. *Anti-sera*

Rabbits were injected biweekly for 14 weeks with 20 mg of crude megacin A preparation (a dialyzed solution of material precipitated at an ammonium sulfate concentration of 50–60% saturation) in incomplete Freund's adjuvant. The rabbits were bled 1 week after the final injection and the sera were stored at −20°C. The γ-globulin fraction of the sera was prepared by the method of FAHEY and HORBETT (1959).

6. *Thin layer chromatography (TLC)*

Silica gel G (Merck) plates were used for TLC. Phospholipids were applied to the plates and developed with the solvent diisobutylketone-acetic acid-water (8 : 5 : 1).

7. *Protein determination*

For protein determination, the biuret method was used, or a Hitachi spectrophotometer (Type EPU-2A) was used for measurements of absorbancy at 280 mμ.

8. *Chemicals*

N-methyl-N'-nitro-N-nitrosoguanidine (NG) was obtained from the K & K Laboratory, (New York, U.S.A.). NG was freshly dissolved in water before use, and, if necessary, sterilized by passage through a Millipore filter. Lecithin was prepared from egg yolk phospholipids by alumina column chromatography as described by SINGLETON *et al.* (1965), and lysolecithin according to HANAHAN (1952).

RESULTS

1. *Assay method for phospholipase A in hemolytic activity*

As evidence has accumulated suggesting the identity of megacin A and phospholipase A, and since phospholipase A can lyse red blood cells upon the addition of phospholipids, the hemolytic activity of the crude UV-induced lysate of strain 216 containing megacin A was examined. For the assay, 10 ml of 1 per cent Difco agar Noble dissolved in 1 per cent NaCl containing 0.06 per cent $CaCl_2$ and 0.02 per cent egg yolk lecithin were poured into Petri dishes. After solidifying, several holes were punched out and a small amount of soft agar containing 1 per cent NaCl was poured into each hole to cover the bottom. To the holes,

0.05 ml aliquots of serial dilutions of megacin A preparation were added and kept overnight; then the plate was covered with 3 ml of soft agar dissolved in 1 per cent NaCl containing about 7×10^8 sheep red cells; the plate was kept at room temperature for 4 hours. Hemolysis could be seen around those holes to which more than 125 units of megacin A were added. Although the sensitivity of phospholipase A assay was not of the same order as that of the megacin A assay on the most sensitive indicator strain KM, it was more sensitive than that of the megacin A assay using the megacinogenic strain 216.

When either lecithin or Ca^{++} was omitted, no hemolysis or a very weak hemolytic activity could be seen. When EDTA was added to the agar or to the megacin preparation, no hemolysis was found. From these results we concluded that the hemolytic activity of megacin A was probably due to phospholipase A. The units of activity were calculated from the highest dilution (Dil) giving hemolysis around the hole as follows:

$$\text{phospholipase A units per ml} = \frac{1}{0.05\times \text{Dil}}.$$

2. *Induction of megacin A by NG*

The induction of phage or bacteriocin by N-methyl-N′-nitro-N-nitro-soguanidine (NG) was described by IVÁNOVICS and his colleague (1964, 1965). The authors found that the maximal titer of megacin A obtainable by NG induction was greater than by UV induction hence NG was used in this study.

An overnight culture of *B. megaterium* strain 216 in medium I was diluted 1 : 100 in fresh medium and incubated with shaking at 37°C. When the optical density of the culture reached 0.1, NG was added to a final concentration of 10 μg per ml and the mixture incubated further. The optical density continued to increase for about 60 minutes and then decreased. At 20 minute intervals, samples were taken and centrifuged. The washed cells were resuspended in saline to the original volumes and lysed by sonication at 10 Kc for 3 minutes.

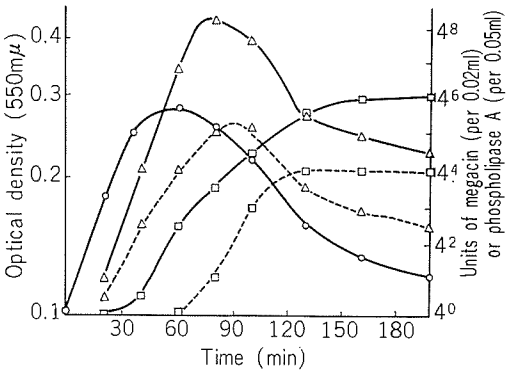


FIGURE 1 Time course of production of megacin A and phospholipase A after NG induction.
○———○ Optical density of culture in a Coleman junior spectrophotometer.
△———△ Intracellular megacin activity.
△·····△ Intracellular phospholipase activity.
□———□ Extracellular megacin activity.
□·····□ Extracellular phospholipase activity.
NG was added at time zero.

The supernatants and cellular lysates were assayed for both megacin A and phospholipase A activity. The results are shown in Fig. 1.

As can be seen, the extracellular megacin and phospholipase activities increased in parallel; and, prior to the release of these activities from the cells, higher activities were found in the cellular lysates, as ALFÖLDI (1958) reported. It is obvious that the concomitant emergence of both activities took place after induction. The intracellular synthesis curves are not in parallel in the early phase of induction (until 40 minutes). The hemolytic zones of the samples taken for the initial 40 minutes were somewhat different in appearance, and this might be due to other hemolytic substance(s) released from the cells. The culture lysate of the control tube was assayed at 180 minutes; only 50 units per ml of megacin were detected and no phospholipase was found.

3. *Parallelism of megacin A and phospholipase A activities at each purification step*

As both megacin A and phospholipase A activities were induced by NG, it is possible that megacin A is a specialized expression of

TABLE 1 *Specific activities of megacin A and phospholipase A at each purification step*

	Protein mg/ml.	Specific activity		Ratio of activity megacin/ phospholipase
		Megacin ($\times 10^5$)	Phospholipase ($\times 10^4$)	
1. Crude lysate	2.4	0.42	0.21	20
2. Isoelectric precipitation	3.4	3.0	1.5	20
3. Am_2SO_4 precipitation	6.2	5.2	2.6	20
4. DEAE-cellulose 0.3 M fraction	10.5	9.8	4.9	20

phospholipase A. To investigate this further, both activities were measured at each step of the megacin A purification procedure described by HOLLAND (1961) for: *i.e.* 1) crude lysate after induction; 2) the precipitate, obtained by isoelectric precipitation at pH 4.0 adjusted with 1 N acetic acid, and dialyzed against 5 mM phosphate buffer (pH 7.0); 3) the precipitate, salted out between 50 and 60 per cent saturation of ammonium sulfate, and dialyzed against the same buffer; 4) the eluate, obtained with 0.3 M NaCl in the stepwise chromatography of DEAE-cellulose column at constant pH and concentration (5 mM) of phosphate buffer, then dialyzed and concentrated.

The results are shown in Table 1. As can be seen, both activities were purified in parallel.

4. *Ultracentrifugation*

According to HOLLAND (1961), the preparation of megacin A purified by DEAE-cellulose chromatography showed a single peak in ultracentrifugation with a molecular weight of 38,000.

In order to see whether megacin A and phospholipase A activities can be separated, ultracentrifugation of the highly purified preparation was performed in a sucrose-density gradient (5–20%) using an SW-50 rotor (Beckman L2) at 40,000 rpm for 19 hours at 4°C. After the run, two drops were collected in each tube, and both activities and protein content were assayed. As shown in Fig. 2, only a single protein peak (absorbancy at 280 mμ) could be seen and, in addition, the peaks of

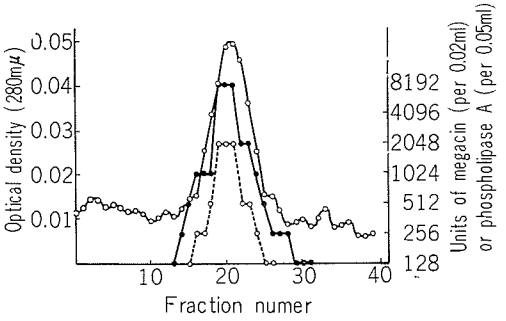


FIGURE 2 Ultracentrifugation of megacin A.
○——○ Optical density.
●——● Megacin activity.
○- - -○ Phospholipase A activity.

both activities were superimposed on that of the protein peak. The result indicates that both active proteins are of the same molecular weight.

5. *Heat stability*

To investigate the probable identity of megacin A with phospholipase A, the rate of heat inactivation was compared. The highly purified preparation of megacin A was diluted with 5 volumes of M/20 phosphate buffer at pH 7.05 and heated in a boiling water bath. Samples were taken at intervals and immediately chilled in an ice water bath and assayed for both activities.

As can be seen in Fig. 3, both activities decreased in parallel.

The heat-stability at various pH's was also studied. A highly purified preparation was diluted with 10 volumes of 5 mM phosphate

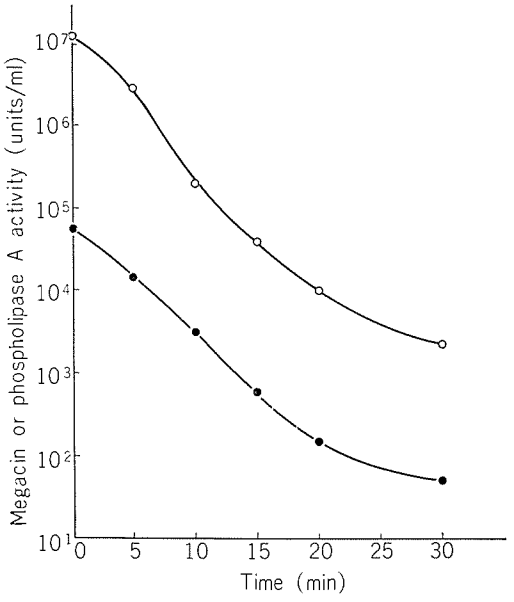


FIGURE 3 Heat inactivation of megacin A and phospholipase A activity.
○——○ Megacin A activity.
●——● Phospholipase A activity.

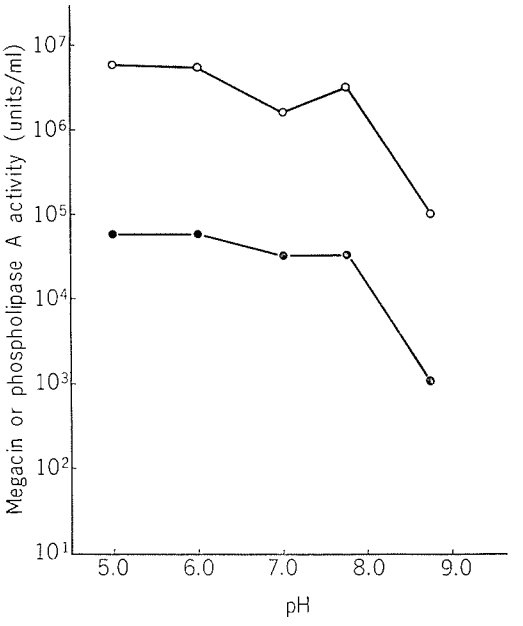


FIGURE 4 Heat stability of megacin A and phospholipase A activity at various pH's.
○——○ Megacin A activity.
●——● Phospholipase A activity.

buffer at pH 7.05 and then dialyzed against 5 volumes of M/20 acetate (pH 5.05), phosphate (pH 6.05 and 7.05) and borate (pH 7.75 and 8.75) buffers, respectively. The dialyzed preparations were heated in a boiling water bath for 5 minutes, immediately chilled and assayed for residual activities. As shown in Fig. 4, megacin A and phospholipase A were more stable in the acid pH range.

6. *Protoplast-lysis by megacin A*

Since the above described results indicate the identity of megacin A with phospholipase A, and since the phospholipase of plakin (HIGASHI *et al.*, 1963, 1966) demonstrated characteristic phenomena in protoplast lysis, *i.e.* a lag period in advance of lysis and the "oxygen effect", protoplast-lysis by megacin A was studied.

The protoplasts of strain KM were prepared with lysozyme in 15 per cent sucrose medium buffered with M/15 phosphate at pH 7.0 and

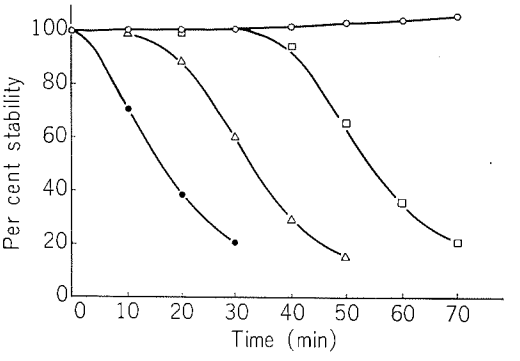


FIGURE 5 Protoplast-lysis by megacin A.
●——● 400 units per ml, final concentration.
△——△ 200 units.
□——□ 100 units.
○——○ No megacin was added.
Optical densities of the tube were measured with a Coleman universal spectrophotometer.

stabilized by adding a final concentration of M/500 Mg^{++}. To all tubes but one, varying amounts of heated megacin A dissolved in 1 ml of 15 per cent sucrose containing the same

buffer were added. As a control, in the last tube, megacin A solution was replaced by buffered sucrose solution. The tubes were incubated at 30°C, mixed at 10 minute intervals by inverting the tubes, and the optical desnity of the tubes was estimated at 10 minute intervals.

When the amount of megacin A was in excess, the turbidity decreased immediately after the addition. However, when smaller amounts were added, lag periods were observed before the turbidity began to decrease, and the duration of the lag period was inversely proportional to the amount added (Fig. 5).

When tubes containing the protoplast suspension in a sucrose medium with added megacin A were left unmixed at 30°C, the turbidity of the suspension decreased more markedly in the top layer than at the bottom. In addition, when the experiment was performed in a Thunberg tube under anaerobic conditions, the turbidity began to decrease evenly at all depths of the tube; furthermore, the lag period was much longer as compared to that of the aerobic experiment of the same composition (" the oxygen effect "), where the tube contents were mixed by inversion at 10 minute intervals (Fig. 6).

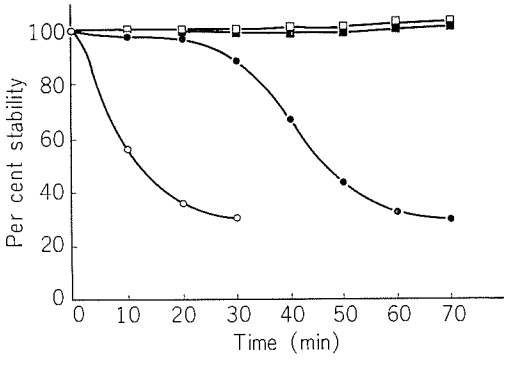


FIGURE 6 " Oxygen effect " in protoplast-lysis by megacin A.
○——○ 1,500 units per ml, aerobic.
□——□ No megacin, aerobic.
●——● 1,500 units per ml, anaerobic.
■——■ No megacin, anaerobic.
Optical densities were measured with a Coleman junior spectrophotometer.

7. *Megacin A negative mutants*

If megacin A and phospholipase A are distinct entities, the majority of the megacin A negative mutants would still produce phospholipase A. To clarify this problem, megacin A negative mutants were isolated.

A suspension of 10^8 spores of the megacin A-producing parent was spread on each nutrient agar plate and heavily irradiated with UV to give about 100 survivors. After overnight incubation, replica plates were taken, and the original plates were covered with soft agar containing about 10^7 cells of the parent strain, then again incubated. As the parent strain 216 is also sensitive to the self-produced megacin A, although to a lesser degree than strain KM, the inhibition zones were restricted to about 2 mm in radius. The megacin A negative mutants were obtained from the replica plates and re-examined for megacin A production using strain 216 as indicator.

Thus six new mutants were obtained and again examined for the absence of megacin A using strain KM, the most sensitive indicator. All of them gave an inhibition zone of growth; however, the appearance of the zone differed from that of the parent. The edge of the zone was not clear-cut, whereas that of the parent was quite clear-cut. The bactericidal action of the megacin A preparation was neutralized by its antiserum when added to assay plates, whereas the megacin produced by the mutants was not neutralized at all and no reduction of the inhibited zone was demonstrated. As HOLLAND *et al.* (1964) described that the other noninducible megacin C is produced by strain 216, the megacin produced by these mutants can be regared as megacin C.

When these megacin A negative mutants were induced by NG, no phospholipase A nor megacin A were produced and, in addition, the production of megacin C did not increase. This means that all these mutants would be double mutants if megacin A and phospholipase A were distinct entities; but such a high frequency of double mutations is incompatible

with the notion that these are independent events. However, it is also possible that a single step mutation had occurred in the gene concerned with the mechanism of induction of both activities.

Attempts were made to find mutants producing cross-reacting material (CRM) to megacin A by adding NG to cultures. In this experiment, Ouchterlony's gel diffusion technique was employed and the precipitation line given by megacin A and its antibodies was defined as described below. No strain showed bacteriolysis by induction and none produced megacin A CRM. In addition, these megacin A negative mutants had become 50–100 times more sensitive than the parent so that their increased sensitivity lay between that of parent strain 216 and strain KM.

8. *Immunochemical evidence for megacin A as phospholipase A*

All the data described above indicate the identity of megacin A with phospholipase A. However, to confirm this identity, immunochemical evidence is required.

The purified preparation obtained by DEAE-cellulose chromatography gave three lines in a gel diffusion experiment (Fig. 7); three lines were also produced in immunoelectrophoresis (Fig. 8), where the antiserum employed was prepared in a rabbit immunized with the preparation obtained by ammonium sulfate fractionation mixed with incomplete Freund's adjuvant. To purify further, the preparation was rechromatographed by DEAE-cellulose in a linear gradient of NaCl at a constant concentration of phosphate buffer (5 mM, pH 7.05). Thus a preparation of megacin A was obtained which contained phospholipase A and gave three precipitation lines.

To obtain an even more purified preparation, this preparation was submitted to zone-electrophoresis with Pevikon in veronal buffer ($\Gamma/2=0.1$) at pH 8.6, 5 volt/cm for 7 hours. After the run, each fraction was assayed for megacin A and phospholipase A activities and also examined for precipitation lines. Phospholipase A activity was always found in fractions containing megacin activities. As the major fractions of megacin gave two precipitation lines and other gave three lines, the former were pooled.

To obtain a preparation giving a single precipitation line, the pooled preparation was heated at 100°C for 10 minutes at pH 5.0 (adjusted with acetate buffer), the precipitate was centrifuged off, and the pH of the supernatant was brought to 7.0. The heated preparation gave only a single line and retained about 50 per cent of both activities.

To show that the undenatured antigenic protein still had both activities, the following

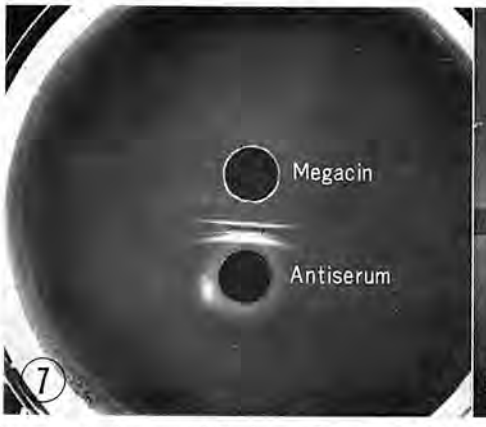


FIGURE 7 Immunodiffusion of a purified megacin preparation.

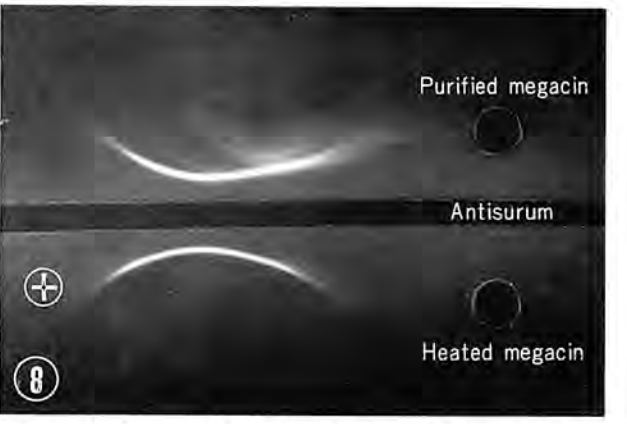
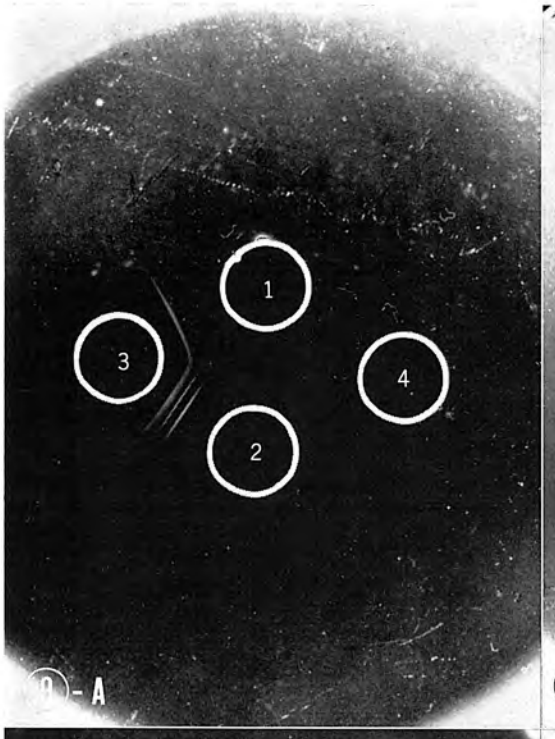


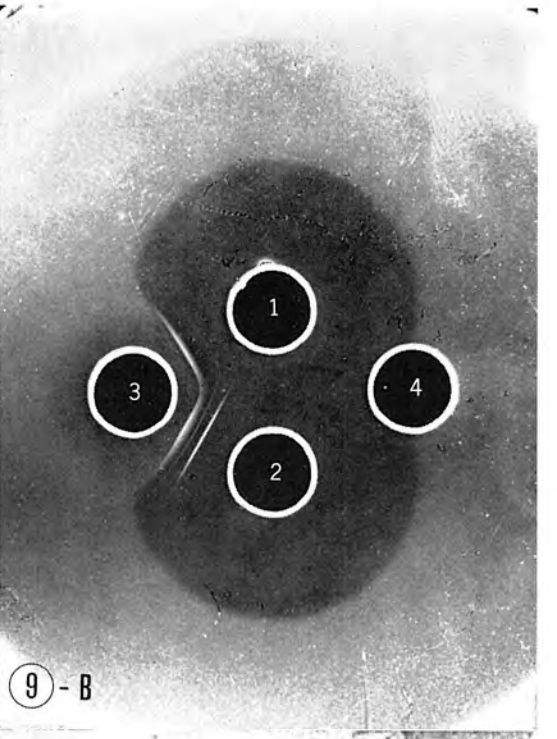
FIGURE 8 Immunoelectrophoresis of a purified and a heated megacin preparation.

FIGURE 9 Effect of anti-megacin serum on the action of megacin.
a. Precipitation pattern in agar gel before the growth of *B. megaterium*.
b. Demarcation of inhibition zone by megacin A on the growth of *B. megaterium*.

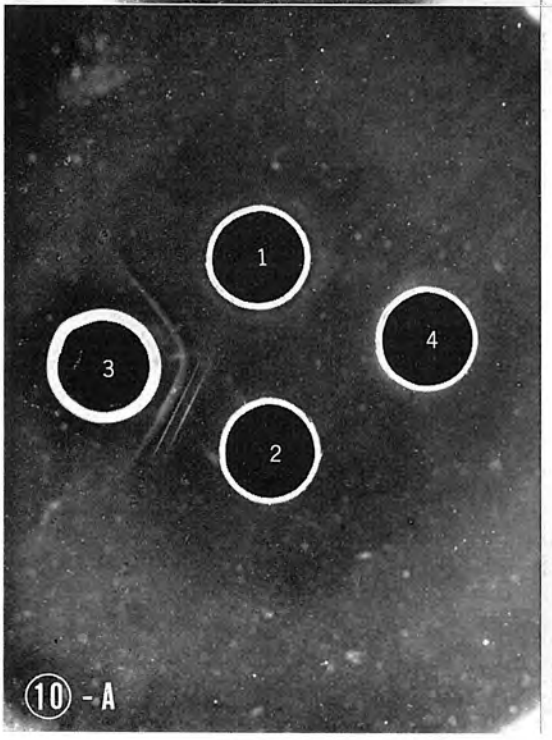
FIGURE 10 Effect of anti-megacin serum on the action of phospholipase A
a. Precipitation pattern before the agar were covered with red cells.
b. Demarcation of hemolyzed zone by phospholipase A.



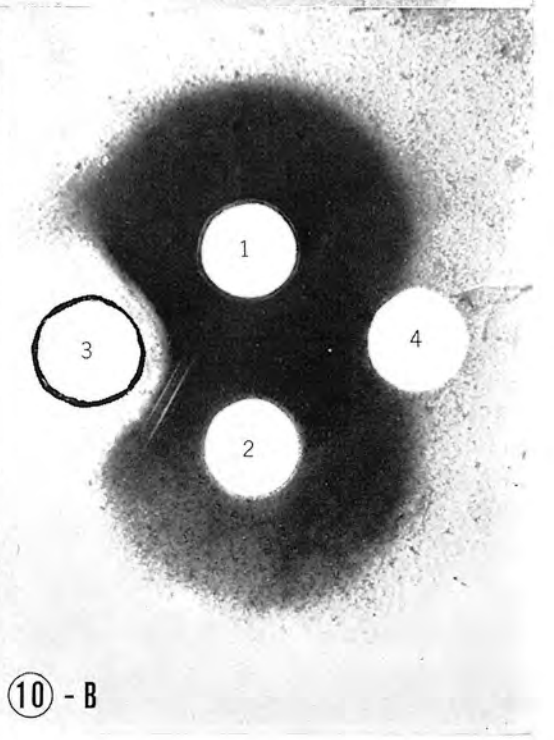
9 - A



9 - B



10 - A



10 - B

two experiments were performed. In one experiment, 10 ml of nutrient agar (1 per cent Difco agar Noble) seeded with about 10^7 cells of strain 216 or KM was poured into a sterile Petri dish; after solidifying, four holes were punched out aseptically as shown in Fig. 9A, and a small amount of sterile soft agar was poured into the bottom of each hole. To holes No. 1 and No. 2, 0.05 ml respectively of the heated megcin A and crude megacin A, obtained by ammonium sulfate fractiontion, were added. To holes No. 3 and No. 4, 0.05 ml respectively of the γ-globulin fraction of megacin A antiserum and the γ-globulin of normal rabbit serum were added. The plate was kept at 4°C for 24 hours. Three lines were found between hole No. 2 and No. 3, whereas only a single line appeared between No. 1 and No. 3 (Fig. 9A). Then the plate was incubated at 37°C for several hours; bacterial growth was inhibited in the area containing diffused megacin A, and that area was delimitted by the precipitation line of megacin A (Fig. 9B).

In the other experiment, 10 ml of 1 per cent Difco agar Noble dissolved in 1 per cent NaCl containing 0.06 per cent $CaCl_2$ and 0.02 per cent egg yolk lecithin were poured into a Petri dish, after solidfying, four holes were punched out, and the same gel diffusion experiments as in Fig 9A. were performed. In this experiment γ-globulins were especially required to remove the nonspecific inhibitor(s) for hemolysis by megacin A contained in rabbit sera. The plate was kept at 4°C for 24 hours and the same pattern of precipitation lines was found as that in Fig. 9A (Fig 10A). Then the plate was covered with 3 ml of soft agat (0.5 per cent Difco agar dissolved in 1 per cent NaCl) containing about 7×10^8 sheep red blood cells, and incubated at room temperature for 4 hours The hemolysis evoked by lysolecithin produced by phospholipase A from lecithin could be seen around the antigen wells, and the area was sharply demarcated by the precipitation line of megacin A (Fig. 10B).

In considering these data, it can be stated that the single antigen contained in the heated megacin A preparation bears both megacin A and phospholipase A activities.

9. *Direct evidence for formation of lysolecithin from lecithin by megacin A*

Since the identity of megacin A with phospholipase A was proved, direct evidence for forming lysolecithin from lecithin must be obtained.

Egg yolk lecithin (0.8 mg) suspended in 0.5 ml of M/20 borate buffer at pH 8.0 was added to 0.1 ml of megacin A containing 5×10^4 units, and incubated at 37°C for 2 hours. The mixture was added to 2.25 ml of chloroform-methanol (1 :2) and, after vigorous shaking, the centrifuged supernatant was evaporated *in vacuo*. The residue was dissolved in a minute

FIGURE 11. Thin layer chromatography of megacin A-treated lecithin.
left: Lecithin control.
middle: Megacin A-treated lecithin
right: Lysolecithin control.

amount of water-chloroform-methanol (1 :1.25 : 2.5) and thin layer chromatography was performed on a silica-gel plate using diisobutylketone-acetic acid-water (8 : 5 : 1) as solvent. In the experiment, a known sample of lysolecithin was also included, and the spots were developed with ninhydrin. As presented in Fig. 12, the reaction product was demonstrated to be lysolecithin.

10. *Effect of megacin A on Bacillus anthracis*

IVANOVICS *et al.* (1959) pointed out the similarity of megacin A and plakin by protoplast-lysis; however, they made reservations on the similarity of the two because megacin A was specific for *B. megaterium* and ineffective on 8 strains of *B. anthracis* (1954) whereas plakin is wellknown to affect several species of the genus *Bacillus* including *B. anthracis*. However, IVANOVICS *et al.* (1955b) reported that 8 of 43 strains of *B. anthracis* were moderately sensitive to megacin A.

To see the effect of megacin A on *B. anthracis*, the following two experiments were carried out.

In one experiment, 0.9 ml of the bacterial suspension of *B. anthracis* strain Vollum in M/20 Tris buffer, pH 8.0, containing M/500 $CaCl_2$ (1×10^7 cells per ml) was mixed with 0.1 ml of the most highly purified (heated) megacin A (8×10^4 units per ml) and incubated at 37°C for 2 hours. As a control, a tube in which megacin was replaced by the same buffer, was included. During incubation, samples were taken and smears were examined after Gram staining under a microscope. As the cells of *B. anthracis* damaged by plakin became Gram-negative after 30 minutes incubation (AMANO *et al.*, 1953), the conversion from Gram-positive to Gram-negative was taken as a criterion for lethal damage of the cells. By 40 minutes incubation, Gram negative cells increased and at 2 hours almost all of the cells were negatively stained. However, when a more dilute preparation was examined, no change could be seen.

In another experiment, the anthracidal activity of the most highly purified megacin A was examined on nutrient agar plates in the same manner as the megacin assay; however no effect was found. Here, attempts were made to improve the culture medium. Into sterile plates were poured 30 ml of 1 per cent agar containing 1 per cent polypeptone (a trypsin digest of casein); the pH of the agar had been adjusted by M/5 tris (hydroxy methyl) aminomethane to pH 7.5. Over the surface were poured 2.5 ml of the medium inoculated with 10^7 cells of *B. anthracis* strain Vollum. The agar also included $CaCl_2$ and varying concentrations of $NaHCO_3$. Serial dilutions of megacin A were made with buffered saline (5 mM phosphate, pH 7.0) and the loopful of each serial dilution was spotted on the agar plate. The plates were then incubated at 37°C for several hours. The medium, con-

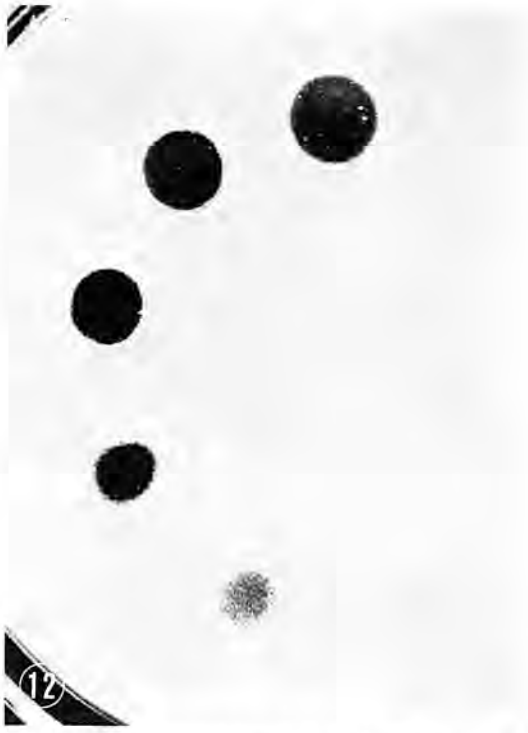


FIGURE 12 Susceptibility of *B. anthracis* to purified megacin A. Two fold serial dilution from an initial concentration of 4×10^5 units/ml.

taining a final concentration of more than 0.2 per cent $NaHCO_3$, enabled megacin A to manifest anthracidal action, with an optimal concentration of $NaHCO_3$ of 0.5 per cent. Fig. 13 demonstrates the anthracidal activity of megacin A. Other strains of *B. anthracis*, Kyoto, Wadayama, NP, Stern and HM were tested with the optimal concentration of $NaHCO_3$ of 0.5 per cent and all were sensitive to the same extent as the strain Vollum. Thus, under specified conditions, megacin A can give lethal damage to cells of *B. anthracis*.

DISCUSSION

Megacin A is an exceptional bacteriocin because bacteriocins, in general, do not evoke cytoplasmic lysis of sensitive bacteria. The cytoplasmic lysis of *B. megaterium* strain KM evoked by megacin A caused us to suspect the identity of megacin A with plakin, the enzymic nature of which was recently suspected to be phospholipase A (HIGASHI *et al.*, 1966). All the data described in this paper support this idea. The most decisive evidence is the results of the gel diffusion experiments with the highly purified megacin A preparation in which a single precipitation line was found. In the antigen excess zone, both magacin A and phospholipase A activities were detected, and in the antibody excess zone both activities were undetectable and both zones were bordered by the gel precipitation line. Although the indirect assay method, hemolysis by formed lysolecithin, was used for the detection of phospholipase A activity in gel diffusion experiments, the purified preparation used was directly proved to hydrolyze egg yolk lecithin giving lysolecithin.

The other problem with regard to the identity was the insusceptibility of *B. anthracis* strains to megacin A, according to IVANOVICS *et al.* (1954). This was solved by our experiment, in which the highly purified megacin A could kill the cells of *B. anthracis* in peptone-agar containing $NaHCO_3$ and Ca^{++}. The apparent activation by $NaHCO_3$ had also been observed on crude plakin (AMANO *et al.*, 1957) and GLADSTON *et al.* (1955) reported that *B. anthracis* grown in a synthetic medium was insensitive to lysozyme and became sensitive when $NaHCO_3$ was incorporated into the medium. Considering these facts and the result of this experiment, the cell walls of *B. anthracis* might be modified to allow megacin A to attack cytoplasmic membranes.

This paper is the first one concerned with the synthesis of an enzyme after NG, UV, or mitomycin C induction. However, this would merely be a special case of induction leading to synthesis of only one or some proteins.

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