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Studies on the Role of Plakin
X. Effect on Membrane Phospholipids

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SUMMARY

The purification of plakin was achieved, after removing the very labile lipoprotein in the crude extract, by ammonium sulfate fractionation and hydroxylapatite column chromatography. Plakin was purified up to 250 fold.

Kinetic studies have shown that plakin itself has no lytic activity on the protoplasts of *B. megaterium* and that "real" lysis is evoked by some lytic agent liberated from spontaneously lyzed protoplasts. During studies on protoplast-lysis by plakin an "oxygen effect" was found. The activity of plakin was reduced in anaerobic conditions or on addition of cytochrome oxidase inhibitors.

Plakin was found to affect the respiratory chain between succinic dehydrogenase and the cytochrome system. However, this is probably a secondary effect due to lysis.

Evidence was obtained suggesting that plakin is a phosphatidase.

INTRODUCTION

In 1907, Gruber and Futaki demonstrated an antibacterial substance active against *Bacillus anthracis* in the blood platelets of the rat, rabbit and horse and termed it plakin. No attempts had been made to elucidate the nature of plakin, until the studies in this laboratory which started about 10 years ago (Amano, Kato and Shimizu, 1952). In our studies some important informations on the mode of action of plakin were obtained using a crude platelet extract: It was found that (1) in addition to *Bacillus anthracis*, *Bacillus megaterium* and various strains of *Bacillus subtilis* are sensitive to plakin (Amano *et al.*, 1952, 1953a); (2) with glutamate, succinate or malate as substrate, oxygen uptake was instantaneously stopped on addition of sufficient plakin (Amano *et al.*, 1952); (3) the permeability barrier of bacteria was damaged at the same time as respiration was inhibited (Amano *et al.*, 1953b, 1956); (4) definite cytoplasmic lysis could be demonstrated only after permeability barrier and oxygen uptake had been affected (Amano *et al.*, 1952, 1953a); (5) for activity against *B. anthracis* but not *B. megaterium* and *B. subtilis* plakin needed two co-factors present in normal serum (bicarbonate ion and a serum protein fraction migrating with albumin or α-globulin) (Amano *et al.*, 1957). To demonstrate the damage in bacterial permeability three experiments were performed: (1) vital stain was used. Intact cells did not stain with neutral red whereas

after treatment with plakin cells were stained within 10 minutes; (2) the intracellular L-glutamate-reserve (Gale, 1947) was estimated. In cells treated with plakin, but not in untreated cells, the reserve was lost by washing (Amano *et al.*, 1953b); (3) plakin lyzed lysozyme-protoplasts of *B. megaterium*, which were otherwise stable, in a sucrose medium (Amano *et al.*, 1956).

Though some aspects of the action of plakin were discovered, its nature as an enzyme or a detergent of protein nature is still obscure. To elucidate this, it was necessary to purify plakin. During purification the activity was assayed as protoplast-lysis and a highly purified preparation was obtained. Working with this purified preparation, evidence was obtained suggesting that plakin is a kind of phosphatidase.

MATERIALS AND METHODS

1. *Bacterial strain used*

Bacillus megaterium strain KM was used. Bacteria were grown at 37°C with mechanical shaking in Penassay broth or in "C" medium, which consists of Na_2HPO_4 (6 gr), KH_2PO_4 (3 gr), NaCl (3 gr), NH_4Cl (2 gr), $MgSO_4$ (40 mg), Na_2SO_4 (110 mg), ferric citrate (34 mg), glucose (10 gr) and sodium glutamate (5 gr) per liter. When P^{32}-labeled cells were needed, the phosphate in the "C" medium was replaced by tris(hydroxymethyl)aminomethane hydrochloride and P^{32}-phosphate was added at a level of 1 μc per ml.

2. *Protoplasts and ghosts*

The bacterial cells were harvested from actively growing cultures suspended in phosphate buffer (pH 7.4) containing 15 per cent sucrose and 2×10^{-3}M $MgSO_4$, and treated with lysozyme. The lysate was prepared by osmotic shock with M/15 phosphate buffer (pH 7.4). The ghosts were obtained by centrifuging the lysate at $14{,}800 \times g$ for 20 minutes.

3. *Phospholipid*

The bacterial cells harvested from the above medium were extracted by chloroform-methanol (1:2) and the extracted phospholipid was separated into chloroform by adding equal volumes of chloroform and 1 per cent KCl solution. The chloroform layer was then washed and evaporated.

4. *Blood platelets*

Platelets were obtained by differential centrifugation of horse or rabbit blood containing 0.5 per cent sodium citrate.

5. *Estimation of protoplast-lysis by plakin*

To tubes containing 5.0 ml of protoplast suspension in 15 per cent sucrose solution, a mixture of 0.3 ml of 50 per cent sucrose solution, 0.2 ml of M/15 phosphate buffer (pH 7.4) and 0.5 ml of varying dilutions of plakin preparation were added, and the tubes were incubated at 30°C. The tubes were mixed by inversion at 10 minutes intervals and, after mixing, the optical densities were read at 550 mμ in a Colman universal spectrophotometer.

6. *Estimation of oxygen respiration*

The oxygen uptake was estimated in a Warburg apparatus or with an oxygen electrode, kindly measured by Dr. B. Hagiwara of the Department of Biochemistry, Osaka University Medical School.

7. *Estimation of succinic dehydrogenase*

This was estimated as oxygen uptake in a Warburg apparatus after the addition of 0.1 per cent phenazine methosulfate and 10^{-3}M of HCN.

8. *Estimation of the difference spectra of cytrochromes*

The difference spectra of cytochromes were kindly measured by Dr. B. Hagiwara in a split-beam spectrophotometer.

9. *Estimation of* P^{32}

A Nuclear Chicago Geiger-Müller counter was used for measuring radioactivity.

10. *Miscellaneous estimations*

Phosphate and protein were determined according to the methods of King (1932) and Kalckar (1947), respectively.

RESULTS

1. *Purification of plakin*

The plakin activity of a crude extract of platelets in saline was readily lost within a few days in a precipitate which formed when the preparation was kept in a refrigerator. The stability of the preparation could be maintained for several days when the crude extract was made with 1 M NaCl solution. As lipoprotein was suspected to be denatured, tests were made to see whether plakin itself was simply lipoprotein or whether it had a distinctive protein nature. For this purpose, a crude extract in 1 M NaCl solution was ultracentrifuged at 50,000 rpm for 3.5 hours in a Spinco model E centrifuge using a J rotor, and the top turbid layer of the lipoprotein fraction and the bottom layer (1 cm from the bottom) were examined for plakin activity. In this experiment plakin activity was found in the bottom layer, and the instability of the preparation was thought to be due to the adsorption of plakin on the denatured lipoprotein which was precipitated.

To obtain a stable preparation tests were made on the effect of removal of the lipoprotein. This was achieved without loss of plakin activity by precipitating the lipoprotein at pH 2.5. The purification procedures used were as follows.

The platelets from 16 l of citrated horse blood were obtained by differential centrifugation, suspended in 500 ml of 1 M NaCl solution and treated in a Kubota sonic oscillator (10 Kc) for 4 minutes. The mixture was centrifuged and the supernatant was adjusted to pH 2.5 with 1 N HCl. The resulting precipitate was removed by centrifugation. The pH of the supernatant was adjusted to 7.0, and plakin was fractionated with ammonium sulfate precipitating between 0.4 and 0.8 saturation. This precipitate was dissolved in 0.1 M phosphate buffer at pH 6.8 and dialyzed against the same buffer. The dialyzed solution was chromatographed on a hydroxylapatite column (Tiselius *et al.*, 1956) and eluted stepwise with 0.1, 0.2, 0.3, 0.4 and 0.5 M phosphate buffer at pH 6.8.

The result of the chromatography is shown in Fig. 2. The plakin activity was eluted with 0.4 M buffer and the main peak of the 0.4 M eluate did not contain

the activity. This means that the purified preparation was still impure. However the purity of the eluate was about 250 times that of the original. It was effective on intact cells of *B. megaterium* and also on cells of *B. anthracis*.

Platelet Suspension in 1 M NaCl soln.
↓ Sonication at 10 Kc for 4 min.
↓ Centrifuge
Supernatant (Fr. 1)
↓ pH 2.5 with 1 N HCl
↓ Centrifuge
Supernatant
↓ pH 7.0 with 1 N NaOH
↓ Centrifuge
Supernatant (Fr. 2)
↓ 0.4-0.8 saturat. with $(NH_4)_2SO_4$
↓ Ppt. dissolved in 0.1 M P-buffer
↓ Dialysis against 0.1 M P-buffer
(Fr. 3)
↓ Hydroxylapatite Column
↓ Stepwise elution with P-buffer
Eluate with 0.4 M P-buffer (Fr. 4)

Fig. 1. Purification of Plakin

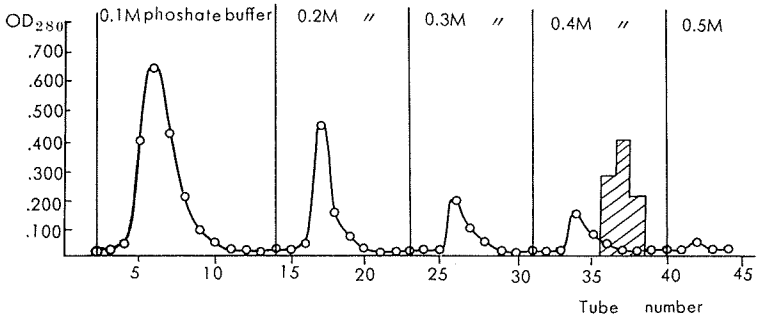

Fig. 2. Hydroxylapatite Column Chromatography of Fr. 3
-○- : Optical density at 280 mμ, Shadowed columns : Plakin activity

As already reported (Asada *et al.*, 1957) esterase and some phosphatases were detected in the crude extract. However they were not detected in the purified preparation using tributyrin, Tween 80, *p*-nitrophenylphosphate, bis (*p*-nitrophenyl) -phosphate as substrates. Moreover, diisopropylfluorophosphate did not inhibit the purified plakin preparation. These facts indicate that plakin is not an esterase, phosphomonoesterase or phosphodiesterase.

2. *Protoplast-lysis of B. megaterium by plakin*

Though the lytic effect of plakin on the lysozyme-protoplasts of *B. megaterium* in a sucrose medium has already been studied (Amano *et al.*, 1956), various kinetic studies were made on the mode of action of plakin. Aliquots of varying dilutions of plakin in a sucrose medium (1:1, 1:2, 1:4) were added to a constant volume of

protoplast suspension. The mixtures were incubated at 37°C, and the decrease in their optical densities were estimated in a Coleman universal spectrophotometer at 550 mμ at 10 minutes intervals. The tubes were mixed by inversion at 10 minutes intervals during the incubation, and the results are shown in Fig. 3. The turbidity of tube No. 1 began to decrease immediately. However those of tubes Nos. 2 and 3 first showed plateaus and then after varying lag periods began to decrease. The three decreasing curves were quite parallel and their slopes showed no relationship to the amount of plakin added, whereas the durations of the lag periods were inversely proportional to the amount of plakin added. As this relationship was always found, the concentration of plakin could be assayed by determining the duration of the lag period. This method was used in determining the purification indices of preparations in the purification procedure described above. The inverse relationship of the lag periods to the concentrations of plakin can be explained by assuming that multiple hits of a definite number are necessary for lysis of a protoplast and that there is a turnover of plakin. The constant rate of true lysis of protoplasts can be explained by assuming that some substances produced from protoplasts through the action of plakin or derived from lyzed protoplasts of very high fragility play a major role in the lysis.

To test this, the plakin activity remaining in the centrifuged supernatant of a protoplast suspension was assayed at varying times during the lag period.

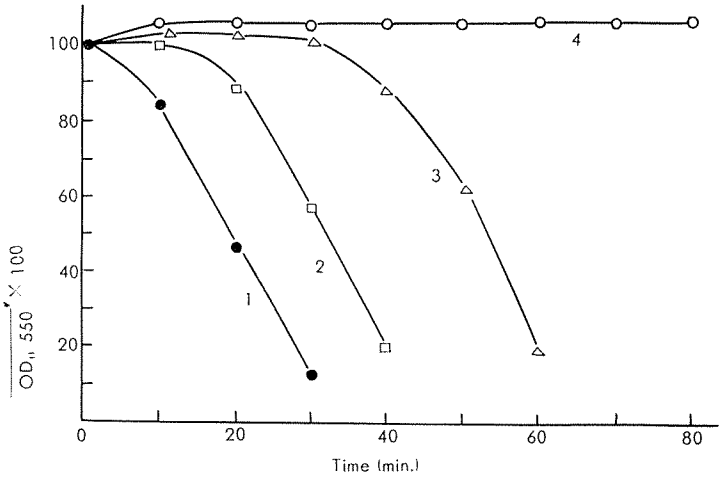

Fig. 3. Protoplast-lysis by Plakin

1 : Tube No. 1 (plakin dilution 1 : 1)
2 : Tube No. 2 (plakin dilution 1 : 2)
3 : Tube No. 3 (plakin dilution 1 : 4)
4 : Tube No. 4 (without plakin)

Aliquots of varying dilutions of a plakin preparation (1:1, 1:2 and 1:4) made with 15 per cent sucrose solution containing M/15 phosphate buffer were added to three tubes containing the protoplast suspension in the sucrose buffer medium. These tubes served as references for plakin activity (tubes R-1, R-2 and R-3). A control tube without added plakin was also included (tube C). Three experimental tubes, the contents of which were identical with that of R-3, were incubated at the same time, mixed at 10 minutes intervals by inverting the tubes, and the optical densities of the four tubes (R-1, R-2, R-3 and C) were estimated. The experimental tubes were centrifuged after 5 minutes (E-1), 10 minutes (E-2) and 20 minutes (E-3), and their supernatants were obtained (S-1, S-2 and S-3). The plakin activity in these three supernatants was assayed. For the assay, 5 ml of each supernatant were added to tubes containing protoplast suspensions. The results are shown in Fig. 4.

The supernatant (S-3) obtained at the beginning of true protoplast-lysis exhibited stronger activity than the other two (S-1 and S-2). S-1 and S-2 contained almost half the original plakin activity. This indicates that supernatant (S-3) obtained at the beginning of protoplast-lysis contained another substance liberated from 2 or 3 per cent of the protoplasts already lyzed.

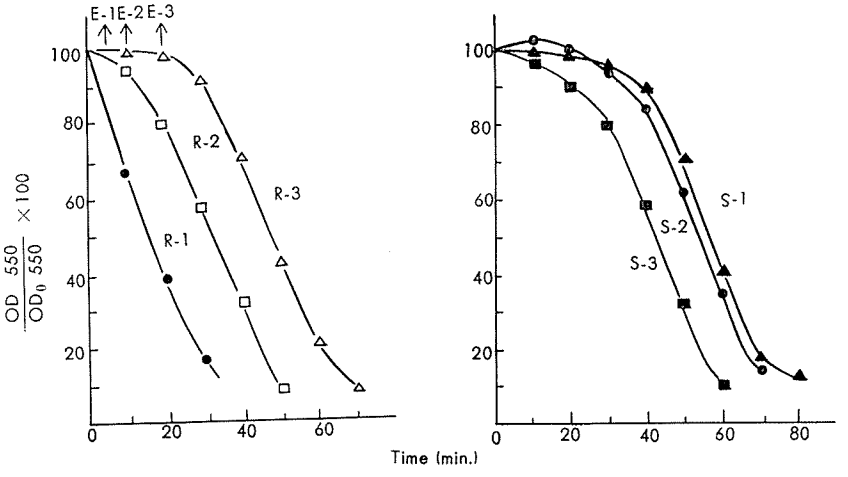

Fig. 4. Assay of Plakin Remaining in Supernatants During the Lag Period

To demonstrate the accelerative effect a substance in protoplast-lyzates on the activity of plakin, the following experiment was performed.

The protoplast suspension was prepared with lysozyme in a sucrose medium and 4.0 ml aliquots were distributed in four tubes. To the first tube 1.0 ml of plakin in a sucrose medium was added together with 1.0 ml of the lyzate. To the second only plakin was added and to the third only lyzate. The last served as the protoplast control.

As shown in Fig. 5, the lyzate itself was very weakly lytic, but, when it was added together with plakin, protoplast-lysis occurred more rapidly than with plakin

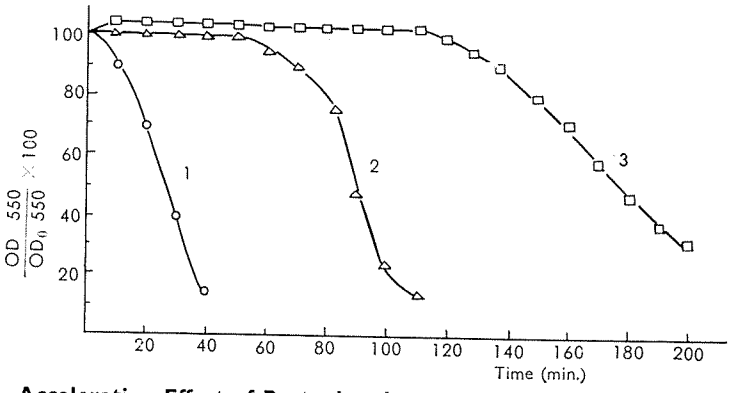

Fig. 5. Acceleration Effect of Protoplast Lyzate on Protoplast-lysis by Plakin

1 : plakin+lyzate
2 : plakin
3 : lyzate

alone. To explain this result, it is suggested that the surface of the protoplasts already attacked by plakin is more susceptible to the lytic agent in the lyzate than that of normal protoplasts. Iványovics reported that, like plakin, megacine, the bacteriocin of *B. megaterium*, could lyze the protoplasts of the indicator strain (1959). Therefore, the identity of the accelerative substance with megacine was studied. The protoplast lyzate was purified by the procedures used for megacine by Holland (1961), i.e. isoelectric precipitation at pH 4.0 and fractionation with between 50 and 60 per cent saturation of ammonium sulfate. The accelerative substance was found in the same fraction with megacine. However, its identity was uncertain at the degree of purification achieved.

3. *Effect of oxygen on protoplast-lysis by plakin*

During studies on protoplast-lysis by plakin, it was occasionally found that, when a tube containing the protoplast suspension with added plakin was not mixed by inverting the tube, the turbidity decreased more markedly in the top layer of the suspension than at the bottom. This might be due to an effect of oxygen. To test this the protoplast suspension was placed in a Thunberg tube, evacuated, and then plakin was added to the suspension. The density decreased at the same rate at the top and bottom of the suspension, but the lag period was very long in comparison with that in aerobic tubes which were mixed at 10 minutes intervals. Moreover, when azide (2×10^{-3}M), HCN (10^{-3}M) or CO was added to tubes under aerobic conditions, very marked lag periods, as in the anaerobic experiments, were observed (Fig. 6). There was no increase in the lag period when azide was added under anaerobic conditions. When the vacuum of a Thunberg tube in an anaerobic experiment was broken during the lag period and the tube was left unmixed, the turbidity decreased rapidly and first at the top of the

suspension. These facts indicate that the protoplast membrane is more susceptible to plakin when electrons are being actively transmitted through the terminal respiratory system.

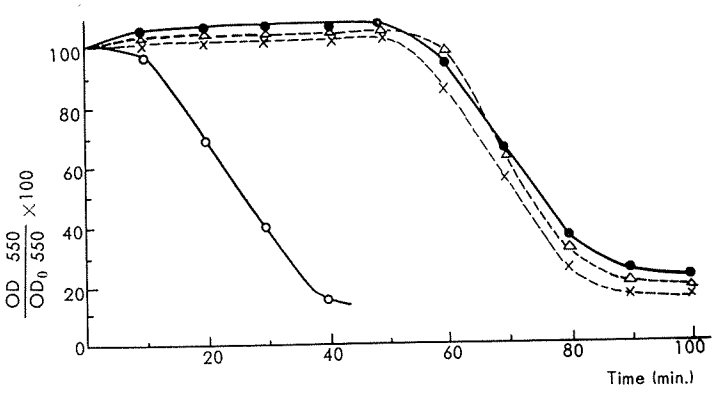

Fig. 6. Comparison of Plakin Activity in Aerobic and Anaerobic Conditions

—○— aerobic condition
—●— anaerobic condition
·····×····· azide
·····△····· HCN

As lipase and desoxycholate lyzed the protoplasts in a sucrose medium, the effect of oxygen in such cases was studied. During protoplast-lysis by lipase, no lag period could be seen and the turbidities of tubes containing varying amounts of lipase began to decrease immediately after its addition at rates varying with the concentration of lipase added. No effect of oxygen could be demonstrated. There was also no lag period before protoplast-lysis with desoxycholate. When varying amounts of desoxycholate above a threshold concentration were added, the optical density decreased very rapidly at varying rates. When less than the threshold concentration was added the protoplasts lyzed very slowly, but without a lag period. These results indicate that the mode of action of plakin is distinct from those of lipase and the detergent, desoxycholate.

4. *Analysis on the cause of the cessation of oxygen respiration*

As plakin attacks the cytoplasmic membranes of the protoplasts and these contain the cytochrome system and various dehydrogenases, the effect of oxygen on protoplast-lysis by plakin can be explained by assuming that some component of the cytochrome system is more easily attacked by plakin when it is in its oxidized form. To clarify this problem, the cause of the cessation of oxygen respiration was studied.

First the parallelism between the cessation of oxygen uptake and protoplast-

lysis was investigated. As protoplasts prepared in a sucrose medium were ruptured by shaking in a Warburg vessel, oxygen uptake was estimated with an oxygen electrode using succinate as a substrate. As shown in Fig. 7, oxygen respiration stopped two minutes after the addition of sufficient plakin and the protoplasts were also lyzed after two minutes. Therefore, protoplast-lysis and cessation of the oxygen uptake occurred in parallel and one did not precede the other.

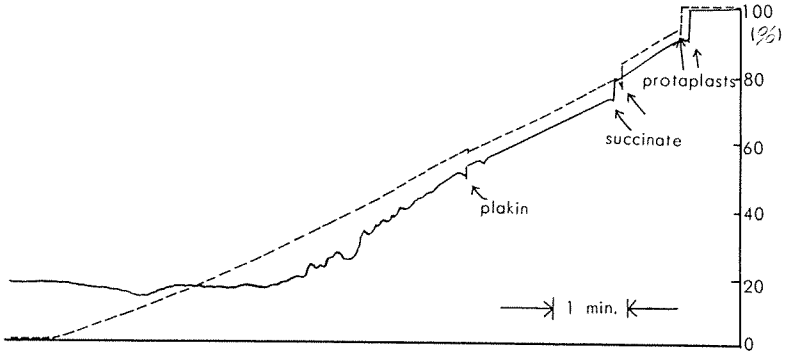

Fig. 7. Oxyegn Uptake by Protoplasts Estimated with Oxygen Electrode
Oxygen uptake was measured in 15% sucrose-0.05 M phosphate buffer. Arrows show added materials.

Studies were made on the location of the block in the respiratory chain caused by plakin. Though the succinic dehydrogenase of the lyzate was once found to be intact when assayed in a Warburg vessel with added HCN and methylene blue (Amano *et al.*, 1958), this result could not be reconfirmed in repeat experiments. Therefore, the enzyme activity of the lyzate was again studied in a Warburg vessel using phenazine-methosulfate (final concentration 0.1 per cent) as an electron-transmitter to oxygen in the presence of HCN. In this experiment succinic dehydrogenase was very active even in a plakin-lyzate of the protoplasts, as shown in Fig. 8. Therefore, the block of the respiratory chain in the oxidation of succinate seems to be located between succinic dehydrogenase and cytochrome oxidase. To elucidate this, studies were made on the substrate being oxidized by the cytochrome system in ghosts of protoplasts prepared by lysis with plakin. This substance was found to be DPNH, as shown in Fig. 9. The difference spectra of the cytochromes of the ghosts were studied in a split-beam spectrophotometer. When succinate was added as substrate, no difference spectra of reduced and oxidized forms of the cytochrome components could be detected. However, when DPNH was used as substrate, difference spectra were obtained, as shown in Fig. 10. The same difference spectra were observed when the cytochromes were reduced by sodium hydrosulfite and compared with those in the non-reduced preparation.

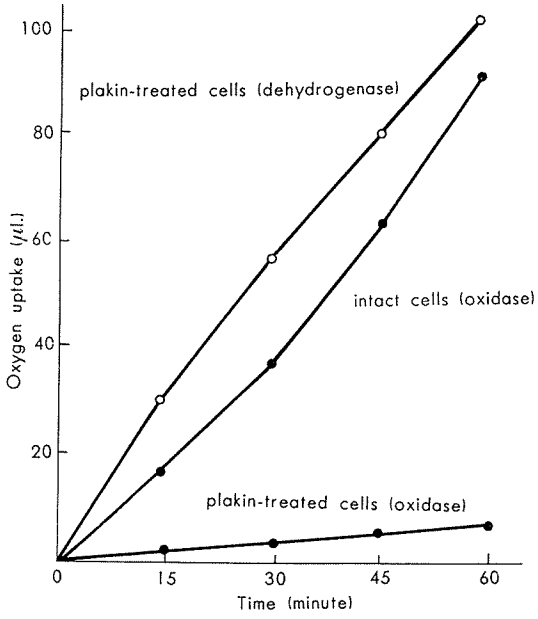

Fig. 8. Succinic Oxidase and Dehydrogenase Activity of Plakin-treated Cells

B. megaterium was treated with plakin in side arm of Warburg vessel. Succinic oxidase activity and dehydrogenase activity were measured by manometric method. Oxidase activity (●——●) was measured with 0.01M succinate. Dehydrogenase activity (○——○) was measured with 0.01M succinate, 0.1% phenazine methosulfate and 0.001 M HCN.

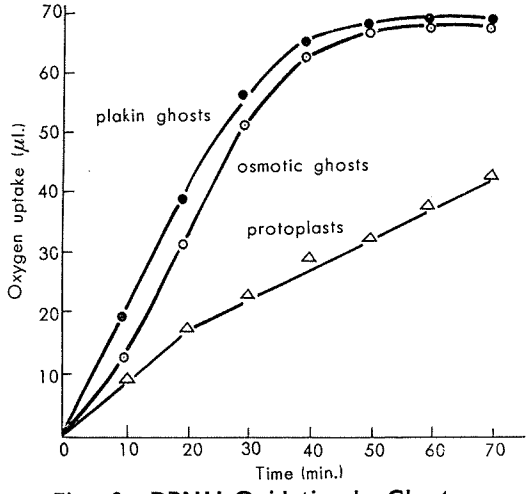

Fig. 9. DPNH Oxidation by Ghosts

Protoplast suspension in side arm I was tipped to main chamber ●——●: with plakin 15% sucrose-0.05M phosphate buffer, △——△: with same sucrose-phosphate buffer, and ○——○: phosphate buffer. After 10 min. 6 μM DPNH was added from side arm II and oxygen uptake was measured.

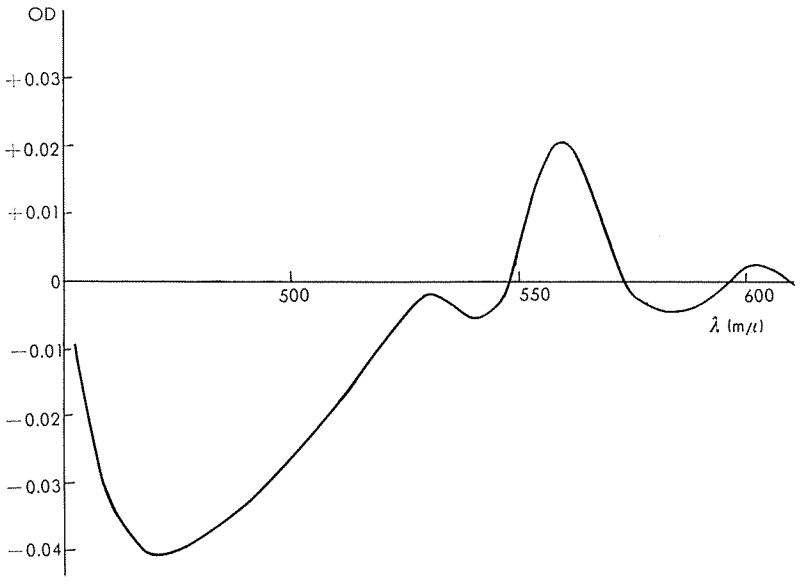

Fig. 10. Difference Spectrum of Ghosts

Freshly prepared ghosts from wet weight 2.4g bacteria were suspended in sucrose-phosphate mixture. Difference spectrum was measured between one half with DPNH and another half without DPNH.

These facts indicate that the block was located not between the cytochrome components but between succinic dehydrogenase and the cytochrome system. However, the same type of block could be found in ghosts of protoplasts prepared by osmotic shock, and the succinic dehydrogenase and DPNH oxidase activities of the ghosts formed by osmotic shock were not affected by further treatment with plakin. From these results it seems that the block in the respiratory chain of succinate oxidation was caused not as the direct action of plakin, but as a secondary effect of some damage to the cytoplasmic membrane by plakin.

5. *Effect of plakin on phospholipid in the protoplast membrane*

As no difference between the activities of the respiratory enzyme system in ghosts formed by plakin and by osmotic shock was detectable, attempts were made of finding a difference in the chemical constitution of these protoplast membranes. Phospholipid was regarded as the most probable substance attacked by plakin, because it is generally regarded as very important for permeability and plakin affected the permeability of the cytoplasmic membrane. To investigate the effect of plakin on phospholipids, p^{32}-phospholipids were used as substrates for plakin.

Table 1. Contents of the Reaction Mixtures

	1	2	3	4	5	6
	ml	ml	ml	ml	ml	ml
Plakin	1.0	1.0	1.0	0.5	0.25	
Borate buffer				0.5	0.75	0.5
P^{32}phospholipid emulsion	1.0	0.5	0.25	1.0	1.0	0.5
Water	1.0	1.5	1.75	1.0	1.0	0.5

Plakin : dialized against borate buffer
Borate buffer : M/10 borate buffer (pH 8.5)
P^{32}-phospholipid emulsion : 50,000 cpm/ml

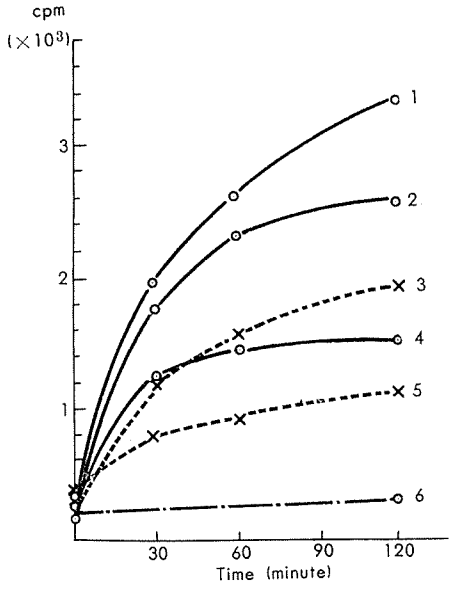

Fig. 11. Effect of Plakin on Membrane Phospholipid: Increase of Breakdown Products Containing P^{32} in Methanol-Water Fraction

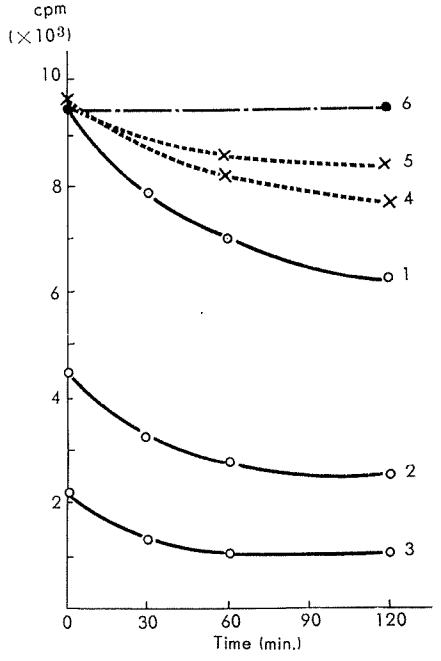

Fig. 12. Effect of Plakin on Membrane Phospholipids: Decrease of P^{32}-phospholipid in Chloroform Fraction

The contents of tubes in this experiment are shown in Table 1. Tubes 1, 2 and 3 show the effect of varying concentrations of phospholipid with a constant amount of plakin and tubes 1, 4 and 5 demonstrate the effect of varying concentrations of plakin with a constant amount of phospholipid. Tube 6 served as control as it contained no plakin. The tubes were incubated at 37°C for 2 hours and 0.6 ml samples were taken from each tube (except tube 6) after 0, 0.5, 1 and 2 hours. Samples were taken from tube 6 after 0 and 2 hours. These samples were fractionated according to Gibson's procedure to separate the methanol-water (KCl) and the chloroform layers. To each 0.6 ml sample 2.25 ml of a chloroform-methanol (1:2) mixture were added and the mixtures were shaken well and centrifuged. To the supernatant, 0.75 ml aliquots of chloroform and 1 per cent KCl solution

were added and the mixtures were again shaken well and centrifuged. One ml of the methanol-water layer containing breakdown products and 0.5 ml of the chloroform layer containing unchanged phospholipids respectively were assayed in a Nuclear Chicago Geiger-Müller counter. The counts observed in the methanol-water layer are shown in Fig. 11 and those in the chloroform layer in Fig. 12.

As can be seen in these figures, the chloroform soluble phospholipid decreased and conversely the breakdown products containing phosphate increased during the reaction. Curve 3 in Fig. 11 shows that a maximum of 55 per cent of the added phospholipid was broken down by the given amount of plakin and the results of both figures indicate that plakin has an enzymatic effect on phospholipid. In addition, results show that ghosts obtained osmotically still contain some phospholipid as substrate for plakin.

Further studies were made on the effect of plakin on unlabeled membrane phospholipid and on the optimal pH for plakin activity.

The phospholipid was extracted from ghosts formed by osmotic shock with a chloroform-methanol (1:2) mixture according to the method of Gibson *et al.* (1961). A turbid emulsion of phospholipid in water (3 mg per ml) was made by evaporating the chloroform from a mixture of 5 ml of chloroform layer and 10 ml of distilled water. To tubes containing 0.3 ml of 0.2 M borate buffer at varying pH values (7.2, 7.6, 8.0, 8.2, 8.5 and 9.0), 0.4 ml of lipid emulsion and 0.3 ml of plakin preparation were added. The tubes were incubated at 37°C for 1 hour, and then the contents of each tube were mixed with 3.75 ml of chloroform-methanol (1:2) mixture. The resulting methanol-water layer was dried and the residue was hydrolyzed with perchloric acid according to the method of King (1932). After hydrolysis, the free phosphate was estimated.

As can be seen in Fig. 13, the non-labeled phospholipid was also found to be hydrolyzed by plakin and the optimal pH for this was 8.5. In the tube at pH 8.5,

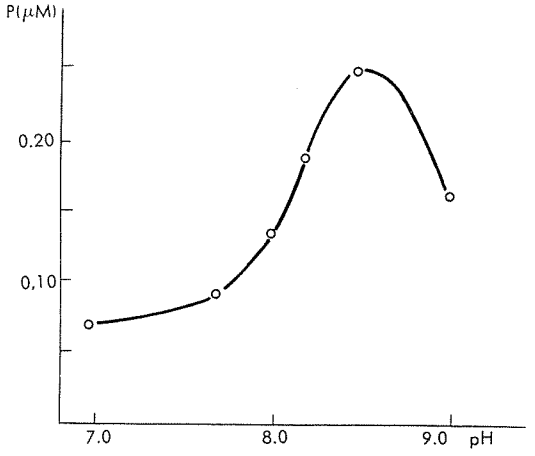

Fig. 13. pH Optimum of Plakin Activity on Membrane Phospholipids

the possibility of an increase in free phosphate was investigated, but no increase was detected and the amount present was negligible. Though results are not

given in this paper, the phospholipid of the cytoplasmic membrane was also split by plakin in the same manner as when ghosts containing P^{32}-labeled phospholipid prepared by osmotic shock were incubated with plakin. These results show that plakin hydrolyzes some phospholipid in the cytoplasmic membranes of sensitive bacteria.

DISCUSSION

Although the mode of action of plakin has been studied for more than ten years, its purification had not been attempted until the last few years because of the meager yield of platelets obtained from blood. Without purifying plakin, no progress was made in studies on its nature. Therefore, its purification was undertaken. At first, this met with two difficulties. The one was the low yield of blood platelets from blood. However this was partially overcome by using horse platelets. The other was that the activity of the preparation was rapidly lost on precipitates of denatured material. This was overcome by precipitating the labile lipoprotein at pH 2.5 in 1.0 M NaCl. This separation permitted the further purification of plakin by salting out with ammonium sulfate and by hydroxylapatite column chromatography. This last procedure was very efficient for raising the specific activity, but the yield of plakin was very low (10-20 per cent). As can be seen in Fig. 2, the purified preparation must still be impure, because plakin activity was eluted after the main peak of the protein in the eluate. Further purification of this preparation was impossible because of the poor protein yield.

The previous report from this laboratory (Amano *et al.*, 1956), was on protoplast-lysis of *B. megaterium*. However, in the previous experiments enough plakin was always used to lyze the protoplasts and hence the lag period before lysis was not detected. The lag period observed on addition of dilute plakin was a surprising phenomenon and a similar lag period was not seen during lysis of protoplasts with lipase or desoxycholate. The duration of the lag period was inversely proportional to the amount of added plakin but lysis proceeded at the same velocity irrespective of the amount of added plakin. To explain this it seems reasonable to assume that multiple hits on the surface of the protoplasts by plakin are required for lysis and that plakin plays a major role in the lag period but not during "true" lysis. The experiment shown in Fig. 5 further revealed that the surface of the protoplasts after attack by plakin became susceptible to the lytic action of a substance liberated from the lyzed protoplasts. However, the beginning of "true" lysis is still hard to understand if plakin is assumed not to have any lytic power on the protoplasts. To explain this, the authors assume a heterogeneity in fragility of the protoplast population so that only some very fragile protoplasts are spontaneously lyzed after they receive multiple hits by plakin. Then the lytic agent liberated from them lyzes other protoplasts which are ready to be lyzed.

These assumptions can easily explain the fact that no difference in the rate of "true" lysis was observed when varying amounts of plakin were added to the protoplast suspension.

Esterase and phosphatase were previously found in blood platelets (Asada *et al.*, 1957). These enzymes were certainly present in crude platelet extracts (Fr. 1 of Fig. 1). However they were not detected in purified plakin preparations using tributyrin, Tween 80, *p*-nitrophenylphosphate and bis(*p*-nitrophenyl) phosphate as substrates. Although esterase can lyze the protoplasts of *B. megaterium*, as reported by Spiegelman (1958), plakin cannot be considered as an esterase, because esterase had already been lost in Fr. 2 during purification and esterase did not give a lag period even at low concentrations and varying concentrations of esterase began to lyze the protoplasts immediately after its addition but at rates varying with its concentration. In addition, the powerful inhibitor of esterase, diisopropylfluorophosphate, did not inhibit purified plakin.

The "oxygen effect" observed during protoplast-lysis is a phenomenon specific for plakin. As Lehninger (1957) found that the mitochondria from animal cells swelled under aerobic conditions and shrunk under anaerobic conditions even in a cell-free suspension, it is conceivable that the protoplasts also swelled under aerobic conditions and thus the sites susceptible to plakin became exposed. The "oxygen effect" during protoplast-lysis and the instantaneous cessation of oxygen uptake caused by plakin led us to reinvestigate the activities of the members of the bacterial cytochrome system. Succinic dehydrogenase was found to be intact even in "plakin-ghosts" and members of the cytochrome system were also very active when estimated in a split-beam spectrophotometer using DPNH as substrate. Therefore electron transfer from succinic dehydrogenase to the cytochrome system was blocked by plakin. However, the same block was also found in the osmotic ghosts. Two possibilities can be considered from these results, whether the protoplast-lysis by plakin as the primary event and the lysis caused the block in the respiratory chain, or *vice versa*. To investigate this problem, the oxygen uptake by protoplasts was recorded during the action of plakin with reference to protoplast-lysis and it was found that oxygen uptake decreased in parallel with the decrease in turbidity of the suspension. The former possibility was not proved by this experiment, but it was proved by demonstrating the difference in the two types of protoplast-lysis, plakin-mediated and osmotic: the ghosts formed by osmotic shock contained more phospholipid as a substrate for plakin than those formed with plakin.

The effect of plakin on membrane phospholipid suggests that plakin is a kind of phosphatidase. The structure of the substrate and the chemical bond susceptible to plakin are now under study.

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