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Studies on the Rôle of Plakin

IX. Effect on the respiratory activity and on the macromolecular structure of *Bacillus megaterium**.

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SUMMARY

The succinic oxidase activity of *Bacillus megaterium* was markedly decreased by plakin action, but the succinic dehydrogenase activity was enhanced. These results were confirmed by spectroscopic examination of plakin-treated cells in which no electron shift between cytochrome b_1 and a_3 occurred. This indicates that there may be direct or indirect inactivation of Slater's factor.

In ultracentrifugal analysis a new peak was found in a lysate of *Bacillus megaterium* protoplasts obtained by plakin treatment as compared with a lysate obtained by osmotic shock.

INTRODUCTION

As shown in the previous reports (Amano *et al.*, 1952, 1953a, 1953b, 1953c, 1953d), lysis of *B. anthracis*, *B. subtilis* and *B. megaterium* was induced by plakin action, and there was also inactivation of their respiration. Furthermore, the lysis of *B. megaterium* protoplasts also occurred by plakin action in an osmotically controlled medium (Amano *et al.*, 1956).

In this paper to clarify the mechanism of plakin action the effect of plakin on the succinic oxidase and succinic dehydrogenase of *B. megaterium* is analyzed on bacterial cells, protoplasts, ghosts of protoplasts and subcellular fractions.

The effect of plakin on the macromolecular structure of *B. megaterium* is also studied using an ultracentrifuge.

Materials and Methods

1. *Bacteria :*

B. megaterium KM was used. Bacteria were grown in broth at 37°C with mechanical shaking. A 4 hour culture was harvested by centrifugation and

*This report was announced at the 23rd Annual Meeting of the Japanese Bacteriologists in April 1956 at Kumamoto University and at the 8th Symposium in Biological Science in September 1956 at Tokyo University.

washed twice with saline.

2. *Protoplasts and ghosts:*
Protoplasts and ghosts were prepared by Weibull's method (Weibull, 1953a). The protoplasts were prepared by the method shown in Table 1, and all cells became protoplasted within 15 mins. at 30°C. The ghosts were obtained by centrifugation of the lysate at 14,800×g for 30 mins., the lysate was prepared from protoplasts by osmotic shock with 0.03 M phosphate buffer (pH 7.2), or by vibration in a Mickle disintegrator in polyethylene glycol (PEG) controlled medium.

3. *Plakin:*
Plakin was prepared as described in the previous report (Amano *et al.*, 1952)

4. *Determination of succinic oxidase activity:*
Succinic oxidase activity of intact cells, protoplasts and ghosts was determined manometrically. The content of Warburg vessels is shown in Table 1.

Table 1. Content of Warburg vessels for determination of succinic oxidase activity of cells, protoplasts and ghosts

	Cells				Protoplasts				Ghosts			
	A		B		C		D		E		F	
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Cell suspension	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—	—	—	—
Ghosts	—	—	—	—	—	—	—	—	0.5	0.5	0.5	0.5
45% PEG	—	—	—	—	0.5	0.5	0.5	0.5	—	—	—	—
Lysozyme (2mg/ml)	—	—	—	—	0.2	0.2	0.2	0.2	—	—	—	—
Phosphate buffer	1.0	1.0	1.0	1.0	0.3	0.3	0.3	0.3	1.0	1.0	1.0	1.0
Plakin	—	—	0.5	0.5	—	—	—	—	—	—	0.5	0.5
Plakin-PEG	—	—	—	—	—	—	0.5	0.5	—	—	—	—
Phosphate buffer	0.5	0.5	—	—	—	—	—	—	0.5	0.5	—	—
15% PEG	—	—	—	—	0.5	0.5	—	—	—	—	—	—
0.1 M succinate	0.2	—	0.2	—	0.2	—	0.2	—	0.2	—	0.2	—
Phosphate buffer	0.4	0.6	0.4	0.6	0.2	0.4	0.2	0.4	0.4	0.6	0.4	0.6
45% PEG	—	—	—	—	0.2	0.2	0.2	0.2	—	—	—	—

Cell suspension: *B. megaterium* KM (2.5 mg dry wt.)
Phosphate buffer: 0.1 M phosphate buffer (pH 7.2)
Plakin-PEG: Plakin containing 15% PEG.

5. *Determination of succinic dehydrogenase activity:*
Succinic dehydrogenase activity of intact cells, protoplasts and ghosts was determined manometrically. The content of the Warburg vessels is shown in Table 2.

Table 2. Content of Warburg vessels for determination of succinic dehydrogenase activity of cells, protoplasts and ghosts

	Cells				Protoplasts				Ghosts			
	A		B		C		D		E		F	
Cell suspension	ml. 0.5	ml. 0.5	ml. 0.5	ml. 0.5	ml. 0.5	ml. 0.5	ml. 0.5	ml. 0.5	ml. —	ml. —	ml. —	ml. —
Ghosts	—	—	—	—	—	—	—	—	0.5	0.5	0.5	0.5
45% PEG	—	—	—	—	0.5	0.5	0.5	0.5	—	—	—	—
Lysozyme (2mg/ml)	—	—	—	—	0.2	0.2	0.2	0.2	—	—	—	—
Phosphate buffer	1.0	1.0	1.0	1.0	0.3	0.3	0.3	0.3	1.0	1.0	1.0	1.0
Plakin	—	—	0.5	0.5	—	—	—	—	—	—	0.5	0.5
Plakin-PEG	—	—	—	—	—	—	0.5	0.5	—	—	—	—
Phosphate buffer	0.5	0.5	—	—	—	—	—	—	0.5	0.5	—	—
15% PEG	—	—	—	—	0.5	0.5	—	—	—	—	—	—
0.1 M succinate	0.2	—	0.2	—	0.2	—	0.2	—	0.2	—	0.2	—
0.01 M KCN	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
0.01% Methylene blue	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
45% PEG	—	—	—	—	0.2	0.2	0.2	0.2	—	—	—	—
Phosphate buffer	0.2	0.4	0.2	0.4	—	0.2	—	0.2	—	—	0.2	0.4

Cell suspension : *B. megaterium* KM (2.5 mg dry wt.)
Phosphate buffer : 0.1 M phosphate buffer (pH 7.2)
Plakin-PEG : Plakin containing 15% PEG.

6. *Spectroscopic examination :*
The cytochrome bands of normal cells and plakin-treated cells were determined in a Zeiss spectroscope.
7. *Analytical ultracentrifugation :*
Analytical ultracentrifugation of the lysate was done in a Spinco E ultracentrifuge.

RESULTS

1. *Effects of plakin on succinic oxidase activity of B. megaterium.*

It has already been reported that the oxidation of glutamate by *B. megaterium* can be inhibited by plakin (Amano *et al.*, 1953d). The oxidation pathway of glutamate is more complicated than that of succinate, because the dehydrogenase of the latter is a Fe-flavoprotein enzyme and does not require DPN or TPN.

Succinic oxidase activity under the action of plakin was examined on normal cells, protoplasts and ghosts. In Fig. 1, curves A and C show the succinic oxidase activities of normal cells and protoplasts, and curves B and D the succinic oxidase activity of plakin-treated cells and plakin-treated protoplasts. The succinic oxidase activity of cells and protoplasts was inactivated by plakin (curves B and D), and the activity dropped to that of the ghosts (curve E). The activity of ghosts was very weak and no effect of

plakin on the activity of ghosts was observed, which were prepared from protoplasts by osmotic shocking with 0.03 M phosphate buffer. Ghosts were also prepared from protoplasts in PEG controlled medium by a Mickle disintegrator and the effect of plakin was also tested using these ghosts, but no effect was demonstrated. The ghosts, prepared from protoplasts in PEG medium by plakin had the same succinic oxidase activity. In addition, no further lysis of ghosts by plakin was demonstrated.

It is suggested from these results that the succinic oxidase activity retained in the ghosts, prepared by osmotic shocking or by mechanical disintegration in osmotically controlled medium, is only a residual activity and can not be compared to the full activity of intact cells or intact protoplasts.

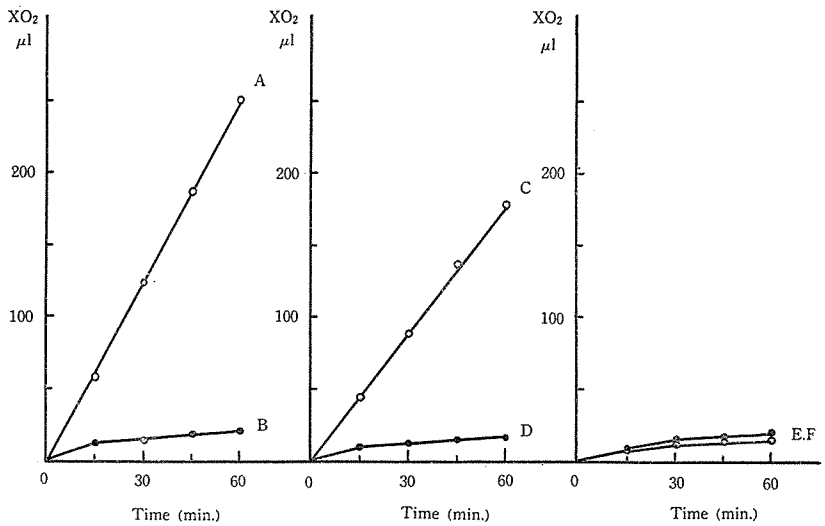


Fig. 1. Succinic oxidase activity of cells, protoplasts and ghosts of *B. megaterium*.
At 15 minute intervals readings of oxygen uptake were recorded.
Conditions are shown in Table 1.
(a) Succinic oxidase activity of cells.
A : Cells.
B : Plakin-treated cells.
(b) Succinic oxidase activity of protoplasts.
C : Protoplasts.
D : Plakin-treated protoplasts.
(c) Succinic oxidase activity of ghosts.
E : Ghosts.
F : Plakin-treated ghosts.

2. *Effect of plakin on the succinic dehydrogenase activity of B. megaterium.*

To analyze the defect in hydrogen transportation of succinate oxidation caused by plakin, the succinic dehydrogenase activity was studied. As shown in Fig. 2, the succinic dehydrogenase activity of normal cells and protoplasts was very weak (curves A and C). However, the activity of cells and protoplasts was greatly enhanced by plakin (curves B and D). The activity of ghosts was also remarkable as shown in curve E and no influence of plakin

treatment was observed (curve F).

It was supposed that the increase in succinic dehydrogenase activity in plakin-treated cells, plakin-treated protoplasts and ghosts might be due to a change of cellular permeability to the methylene blue used in the estimation and that the dehydrogenase activity was not inhibited by plakin. The change of permeability caused by plakin has already been described (Amano *et al.*, 1953 b). The inability of non-toxic dyes to penetrate into protoplast has also been described (Amano *et al.*, 1958). Considering these, the above results can easily be explained.

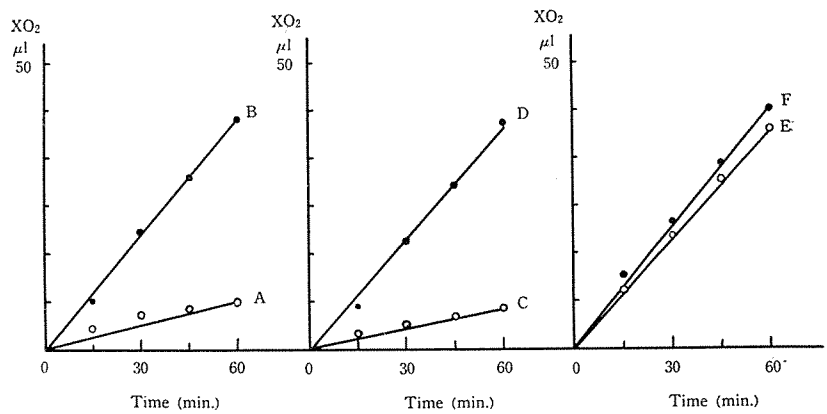


Fig. 2. Succinic dehydrogenase activity of cells, protoplasts and ghosts of *B. megaterium*.
At 15 minute intervals readings of oxygen uptake were recorded.
Conditions are shown in Table 2.
(a) Succinic dehydrogenase activity of cells.
A : Cells.
B : Plakin-treated cells.
(b) Succinic dehydrogenase activity of protoplasts.
C : Protoplasts.
D : Plakin-treated protoplasts.
(C) Succinic dehydrogenase activity of ghosts.
E : Ghosts.
F : Plakin-treated ghosts.

3. *Spectroscopic observation of succinate oxidation :*

It is suggested that the succinic dehydrogenase was not inhibited by plakin. The defect in hydrogen transportation caused by plakin should exist in the terminal respiratory system.

For spectroscopic examination, the cells were prepared as follows ; 4.2 mg. of cells were treated with 10 ml. of plakin at 30°C for 10 mins., cells were centrifuged, washed once with saline and divided in two. One tenth ml. of 0.1 M sodium succinate was added to one part and 0.1 ml. of 0.05 M phosphate

buffer (pH 7.2) was added to the other. Another 4.2 mg. of normal cells were treated similary with 0.05 M phosphate buffer (pH 7.2) in place of plakin. The cells treated with phosphate buffer were used as the controls.

As shown in Fig. 3, the reduced cytochrome bands of normal cells at 554 mμ (b_1) and 590 mμ (a_3) were clearly demonstrated in the presence of the substrate (0.1 M succinate), and faint bands were observed even in its absence. In the plakin-treated cells only a weak cytochrome band at 554 mμ was observed even in the presence of substrate, and no band was observed in its absence.

The cytochrome bands at 554 mμ and 590 mμ were observed when the normal cells and plakin-treated cells were reduced by hydrosulfite. This suggests that cytochrome was not injured or destroyed by plakin treatment. The facts were confirmed by measuring the cytochrome bands of ghosts and of plakin-treated ghosts in a Beckman spectrophotometer according to Weibull's method (Weibull, 1948, 1953b). The same absorption curve was obtained for reduced (by hydrosulfite) and oxidized cytochromes in the ghosts as reported by Weibull (Weibull, 1953b). In addition no difference was found between the absorption spectra of the cytochromes of normal and plakin-treated ghosts. It seems therefore that the disappearance of the cytochrome band at 590 mμ in plakin-treated cells may be due to a defect in electron transport between cytochrome b_1 and a_3 of *B. megaterium* and that an unknown factor or factors play a role in electron transport between cytochrome b_1 and a_3.

If the Slater's factor is present in bacterial cells, the defect of electron shift could easily be explained by its inactivation by plakin.

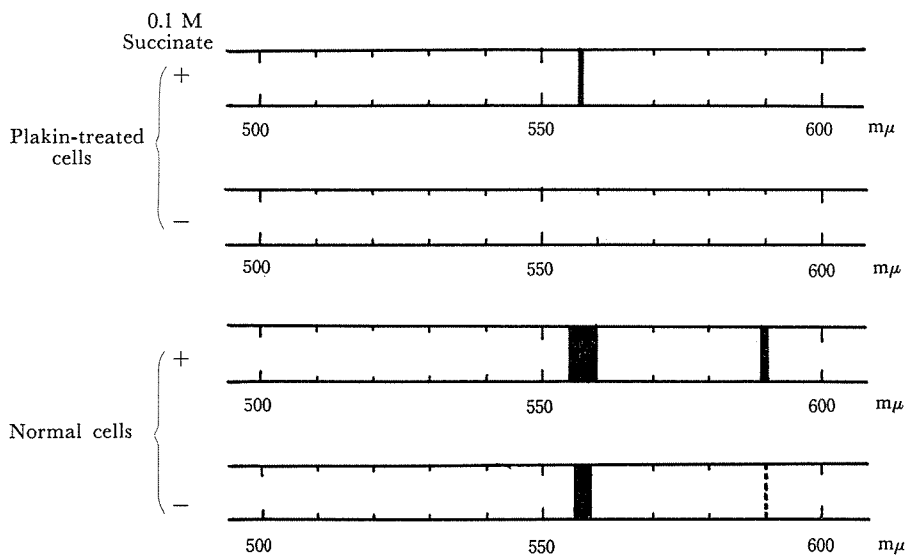
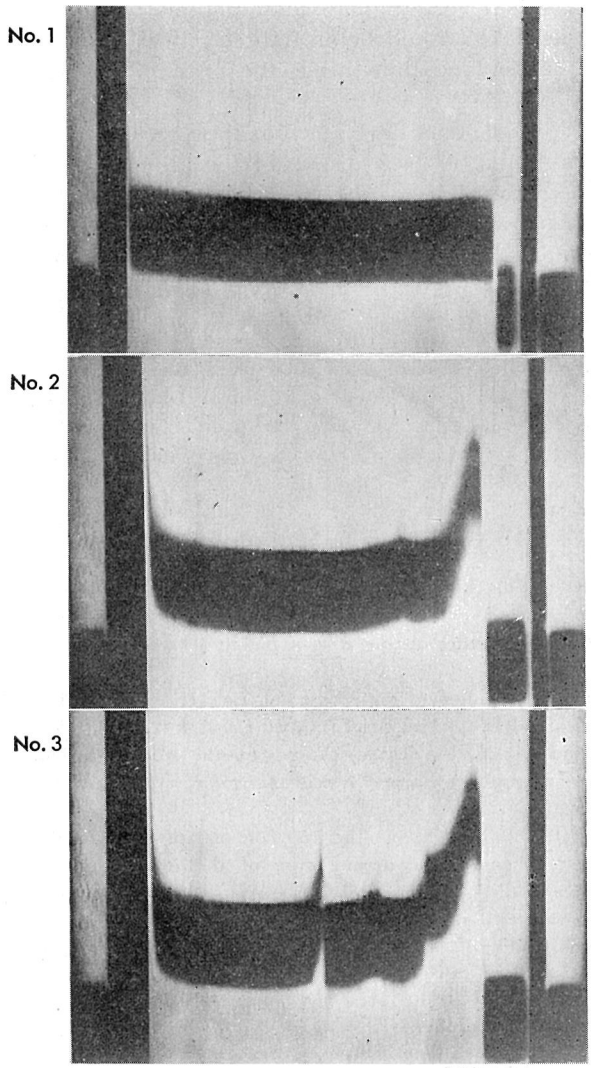


Fig. 3. Spectroscopic examination of cytochrome bands of *B. megaterium*.

4. *The effect of plakin on the macromolecular structure of B. megaterium.*

The cellular components of *B. megaterium* KM were studied by Weibull



(1953b) and four components were found in *B. megaterium* protoplasts lysed by osmotic shock.

To investigate the effect of plakin on the macromolecular structure of *B. megaterium*, the ultracentrifugal patterns of a lysate of *B. megaterium* protoplasts after treatment with plakin was compared with that of the lysate of protoplasts lyzed by phosphate buffer. In Fig. 4, pattern No. 1 indicates that of plakin showing no peak. Pattern No. 2 is that of an osmotically shocked lysate, and No. 3 the pattern of a lysate caused by plakin. As shown in No. 3 a new peak was found, which is not present in the osmotically shocked lysate. The sedimentation constant of the peak was 31.4 S. The nature of the peak is not yet known and now under study.

Fig. 4. Photograph of ultracentrifugal patterns of the plakin and lysates of *B.megaterium* KM.
Experiment was carried out with Spinco E ultracentrifugation at 44, 770 r.p.m. $\theta = 50°$, Temperature $= 14°$, Interval $= 8$ minutes.
No. 1 : Plakin
No. 2 : Lysate prepared from the protoplast by osmotic shock with 0.03 M phosphate buffer (pH 7.2).
No. 3 : Lysate prepared from the protoplast by lyzing with plakin.

DISCUSSION

It was suggested from the above results that inactivation of succinic oxidase of *B. megaterium* may be due to inactivation of the Slater factor or a similar factor and is not due to the direct inhibition of succinic dehydrogenase, cytochrome b_1 or a_3. It is not yet clear whether the defect in the terminal electron transport system caused by plakin is a direct effect, because ghosts prepared from protoplasts by osmotic shocking or by mechanical disintegration

in osmotically controlled medium also had very slight oxidase activity.

We assume that defect caused by plakin would be an indirect effect resulting from a change in the permeability barrier and that this is the primary damage caused by plakin.

Nygaard and Sumner (1953) reported that the succinoxidase of rat liver mitochondria was inactivated by lecithinase A by hydrolyzing the mitochondrial lecithin and that the essential factor for the interaction of succinic dehyrogenase and the remainder of succinoxidase system might be mitochondrial lecithin. It was found in a preliminary experiment that plakin had no lecithinase activity and the inactivation of the Slater factor by plakin may not be caused by the hydrolysis of lecithin.

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REFERENCES

Amano, T., Kato, K. and Shimizu, R. (1952) Studies on the role of plakin. *Med. J. Osaka Univ.*, **3** (2-3), 293-311.

Amano, T., Kato, K and Shima, M (1953a) Studies on the rôle of plakin (II). *Med. J. Osaka Univ.*, **3** (4), 695-710.

Amano, T., Shima, M. and Kato, K. (1953b) Studies on the rôle of plakin. III. The damage of semipermeability of bacteria caused by plakin. *Med. J. Osaka Univ.*, **4** (2-3), 277-284.

Amano, T., Kato, K. Morishima, T. and Okada, K. (1953c) Studies on the rôle of plakin. IV. The effect of plakin on the respiratory enzyme system of *Bacillus megaterium*. *Med. J. Osaka Univ.*, **4** (2-3), 285-289.

Amano. T., Kato, K. and Okada, K. (1953d) Studies on the rôle of plakin. V. On the correlationship of phosphate metabolism to the maintenance of the organization of bacterial cells. *Med. J. Osaka Univ.*, **4** (2-3), 291-297.

Amano, T., Kato, K., Okada, K., Tamatani, Y. and Higashi, Y. (1956) Studies on the rôle of plakin. VII. Effects on the protoplast of *B. megaterium*. *Med. J. Osaka Univ.*, **7**, 217-231.

Amano, T. Kato, K., Tamatani, Y. and Higashi, Y. (1958) Studies on the surface barrier of *Bacillus megaterium*. *Biken's J.*, **1**, 1-4.

Nygaard, Agnar P. and Sumner, James B. (1953) The effect of lecithinase A on the succinoxidase system. *J. Biol. Chem.*, **200**, 723-729.

Weibull, C. (1948) Some chemical and physicochemical properties of the flagella of *Proteus vulgaris*. *Biochim. et Biophys. Acta*, **2**, 351-361.

Weibull, C. (1953a) The isolation of protoplast from *Bacillus megaterium* by controlled treatment with lysozyme. *J. Bact.*, **66**, 688-695.

Weibull, C. (1953b) Characterization of the protoplasmic constituents of *Bacillus megaterium*. *J. Bact.*, **66**, 695-702.