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CHEMICAL STUDIES ON DAMAGES OF *ESCHERICHIA COLI* BY THE IMMUNE BACTERICIDAL REACTION.
I. RELEASE AND DEGRADATION OF PHOSPHOLIPIDS FROM DAMAGED BACTERIA

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SUMMARY When antibody-sensitized *Escherichia coli* B is treated with complement in the absence of lysozyme, bacterial phospholipids or fragments containing phospholipid appear in the surrounding medium. Almost at the start of the reaction, a little phosphatidylethanolamine (PE) appears in the lipid fraction extracted from the supernatant of the reaction mixture. Later it does not increase greatly in amount but free fatty acids (FFA) and lysophosphatidylethanolamine (LPE) appear and increase gradually.

Addition of lysozyme to the reaction mixture enhances the amounts of FFA and LPE released, but does not increase the numbers of the spots on a thin layer chromatogram of the lipid fraction of the supernatant.

The FFA fraction contains no β-hydroxymyristic acid from lipid A of the lipopolysaccharide complex.

INTRODUCTION

In 1968, Inoue et al. showed that complement produces "channels" in the cell wall of antibody-sensitized *Escherichia coli*. Enzymes in the periphery of the cells such as alkaline phosphatase, can easily leak out into the surrounding medium through these channels, while intracellular enzymes, such as β-D-galactosidase, cannot leak out unless the cytoplasmic membrane underneath the cell wall collapses. On the other hand, when lysozyme is present in the surrounding medium it can pass through these channels and degrade peptidoglycan at the base of the cell wall, resulting in formation of spheroplasts.

This and following papers describe studies

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on the chemical substance(s) which is attacked by the complement when the " channels " are produced in the surface structure of sensitized bacteria. In these studies we analyzed labelled substances liberated from ^{14}C-labelled *E. coli* treated with antibody and complement.

MATERIALS AND METHODS

1. *Bacterial strain and culture*

Escherichia coli B/SM, strain 1–1 was obtained by selecting spontaneous mutants from *E. coli* B, strain Hershey. It was maintained on trypticase soy agar slants (TSB agar slants) (BBL, Cockeysville, Md.) or on Tris glucose medium agar slants like those described as Echols et al. (1961) but containing 0.1% glucose instead of 0.2% glucose. Bacteria were labelled by growing them in Tris medium containing 0.03% cold glucose plus 0.5 μCi/ml of ^{14}C-[U]-glucose (specific activity=5.0 mCi/m mole).

2. *Medium*

Physiological saline containing 0.005 M Tris-HCl buffer, 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (TBS^{++}) was used for washing and suspending bacteria and for diluting reagents.

3. *Antiserum and complement*

Rabbits were immunized with heat-killed (56 C for 60 min) *E. coli* B, strain Hershey, which had been cultivated in Tris medium containing 0.1% glucose and then washed. The antiserum was inactivated by heating it at 56 C for 60 min, and then treated three times with bentonite to remove lysozyme activity (Inoue et al., 1959). It was stored at —20 C without any preservative.

Guinea pig complement serum was collected from more than hundred animals. The sera were pooled and centrifuged at 30,000 rpm for 60 min and the floating lipid layer was removed. Lysozyme-depleted complement serum (RL) was prepared by treating the serum with bentonite as described previously (Inoue et al., 1959). Complement serum and RL were stored at —70 C. The hemolytic activity of RL used was 175 to 220 CH_{50}/ml, estimated by Mayer's standard method (1961). Complement was inactivated by heating it at 56 C for 60 min.

4. *Extraction of lipid and thin-layer chromatography (TLC)*

Lipids were extracted by the method of Bligh and Dyer (1959). The chloroform-layer was evaporated in a rotary evaporator. The dried lipids were then redissolved in chloroform-methanol (1 : 1, v/v) and applied to a thin layer of silica gel H or G on a glass plate or to aluminum sheet silica gel (5553) (E. Merck AG, Darmstadt, Germany). Gels were developed with a chloroform-methanol-water mixture (65 : 25 : 4, by vol.). The Rf values of lipids were different with different thin layer systems even using the same solvent system, and the positions of the spots of cardiolipin and free fatty acids were generally reversed when silica gel on glass was used instead of a commercial precoated aluminum sheet.

β-Hydroxyfatty acids were separated from other fatty acid on aluminum sheet silica gel (Merck 5553) with a mixture of heptane and ethylacetate (4 : 1, v/v) or petroleum ether (60–70 C), ether and acetic acid (70 : 30 : 2, by vol.) as described by Braun and Rehn (1969) and Braun et al. (1970).

Fuji X-ray film, medical Kx (Fuji Photo Film Co., Tokyo) was used for autoradiography of TLC.

5. *Measurement of radioactivity*

Samples were dissolved in the scintillation liquid described by Koshland et al. (1970) and radioactivity was measured in a Beckman Liquid Scintillation Counter, LS200B or LS250.

Radiochromatograms were scanned with an Actigraph III, Nuclear Chicago.

6. *Materials*

^{14}C-[U]-Glucose was purchased from Daiichi Pure Chemicals, Tokyo. Fatty acids were obtained from Nakarai Chemicals, Kyoto. β-Hydroxymyristic acid was obtained from Tokyo Chemical Industry, Tokyo. Silica gel H and G and aluminum sheet silica gel (5553) were purchased from E. Merck AG, Darmstadt, Germany. Egg-white lysozyme (3 × crystallized) was obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

1. *Release of ^{14}C from E. coli B/SM during the immune bactericidal reaction*

E. coli B/SM, strain 1–1 was grown in ^{14}C-[U]-glucose with 0.03% glucose-Tris

medium at 37 C for 13 to 16 hr with vigorous aeration. The bacteria were harvested by centrifugation and washed two or three times with cold TBS^{++}. They were then resuspended in cold TBS^{++} at a concentration of 5.0×10^9 bacteria/ml. The suspension was mixed slowly with an equal volume of cold antiserum, optimally diluted in TBS^{++}, in an ice bath with constant stirring. It was then transferred to a water bath of 37 C and shaken mechanically for 15 min. Then it was centrifuged. The sensitized bacteria were washed three times with cold TBS^{++}, and resuspended in the same buffer at a concentration of 2.0×10^{10} bacteria/ml.

One volume of this suspension was incubated in 2.5 volumes of reaction mixture with lysozyme-free complement serum (RL) at a final concentration of 1/20 or 1/25 (A). As a spheroplast forming system (Inoue et al., 1959), egg-white lysozyme was further added to a final concentration of 20 μg/ml (C). As a control, heat-inactivated RL (ΔRL) was added instead of RL (B and D). As another control, the sensitized bacteria were treated with 2 mM tri-sodium ethylenediamine tetra-acetate (EDTA) and 0.5 M sucrose at 37 C for 15 min and then centrifuged. The supernatant (E′) was removed, and the precipitated bacteria were quickly suspended in the same volume of distilled water to cause osmotic shock (E).

After incubation, the reaction mixtures were centrifuged. Parts of each supernatant and precipitate of bacteria was used to measure radio-activity, and other parts were fractionated with chloroform-methanol by the method of Bligh and Dyer (1959).

As shown in Table 1, the radioactivity was released into the supernatant of the immune-bactericidal system, and it increased in both the chloroform phase and methanol-water phase of the supernatant of the system.

The chloroform phase (lipid fraction) was chromatographed on a silica gel plate. Autoradiography of the plate showed spots of phosphatidyl-ethanolamine (PE), lysophosphatidylethanolamine (LPE) and free fatty acid (FFA) as seen in Fig. 1. These spots were more intense using the spheroplast-forming system with excess lysozyme (C) (Table 1 and Fig. 1).

EDTA-sucrose-treatment (E′) followed by osmotic shock (E) caused release of a large amount of ^{14}C into the supernatant, as shown in Table 1. Most of the radioactivity released

TABLE 1. *Percent distribution of ^{14}C of sensitized E. coli B/SM after immune bactericidal reaction or EDTA-sucrose-osmotic shock*[a]

Treated with	A RL	B ΔRL	C RL+ lysozyme	D ΔRL+ lysozyme	E′ EDTA-sucrose	E Osmotic shock
Supernatant (Sup.)	7.23	1.88	10.7	2.23	2.26	32.9
Precipitate (Ppt.)	92.8	98.1	89.3	97.8	—	64.8
Sup.						
Chloroform phase	0.673	0.139	1.07	0.162	0.201	0.218
Methanol-water phase	3.08	0.960	2.80	0.790	1.23	21.2
Ppt.						
Chloroform phase	11.7	12.6	9.07	12.8	—	6.88
Methanol-water phase	2.42	4.33	2.88	4.61	—	3.67

[a] After the reaction, smear preparations showed that about 2/3 to 3/4 of the bacteria in reaction C were converted into sheroplasts while no spheroplasts were observed in other preparations.

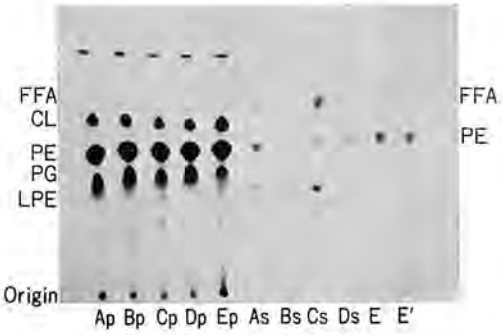


FIGURE 1. *Autoradiogram of thin layer chromatogram of lipid fractions of precipitates and supernatants of the immune bactericidal reaction or EDTA-sucrose-osmotic shock.*

The lipid fractions were the same to those shown as chloroform phase in Table 1. They were applied to silica gel H on a glass plate, and developed with chloroform-methanol-water (65: 25: 4, by vol.). FFA, free fatty acids; CL, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; LPE, lysophosphatidylethanolamine.

See text and Table 1 for samples applied.

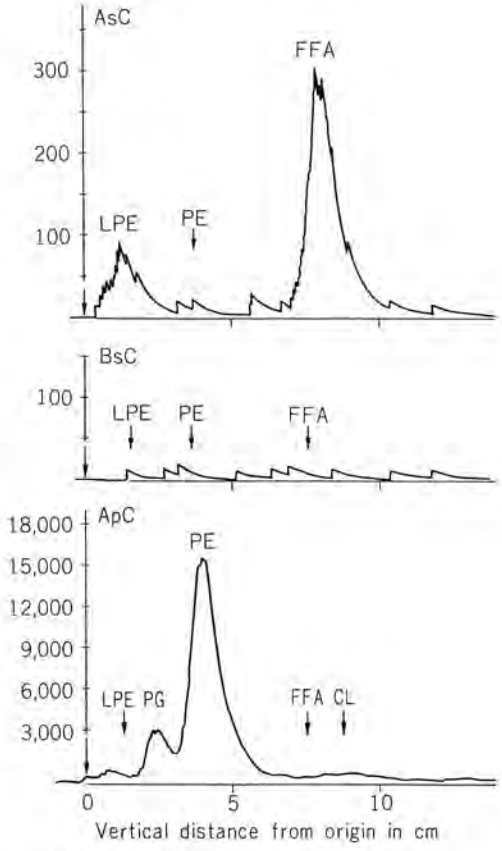


FIGURE 2. *Scans of radioactivity of thin layer chromatograms of the lipid fractions from the immune bactericidal reaction without lysozyme.*

AsC, lipid fraction of the supernatant from the immune bactericidal reaction; BsC: that from a control with inactivated RL; ApC, that of the precipitate from the immune bactericidal reaction. Arrows show the origins for chromatography. The scale of the ordinate for ApC is reduced about 1 to 43. LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; FFA, free fatty acid; CL, cardiolipin.

Chromatography was carried out on a silica gel-precoated aluminum sheet with chroloform-methanol-water (65: 25: 4, by vol.). After autoradiography, the sheet was cut into strips for each sample, and scanned. Note the positions of the spots of CL and FFA were reversed compared with those on silica gel H on a glass plate (see Fig. 1).

was found in its methanol-water phase and interphase. A little PE was detectable in the chloroform phase, but neither LPE nor FFA was observed (Fig. 1).

Fig. 2 shows the scan of a similar radiochromatogram of the lipid fractions from a lysozyme-free bactericidal system.

The lipid fraction of the supernatant contained more FFA than LPE.

2. *Kinetics of ^{14}C-release from bacteria during the immune bactericidal reaction*

Fig. 3 shows the course of the immune bactericidal reaction and the release of isotope into the methanol-water and chloroform phases of the supernatant.

In a similar experiment, aliquots were removed from the reaction mixtures at intervals, and their supernatant and precipitate fractions were each extracted as described above. Fig. 4 shows autoradiograms of these

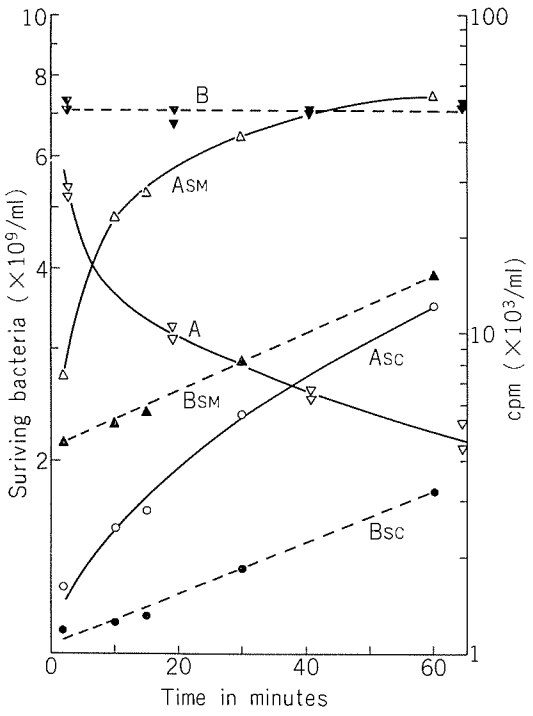


FIGURE 3. *Course of the immune bactericidal reaction and release of ^{14}C into the methanol-water phase and chloroform phase of the reaction supernatants.*

A, immune bactericidal (RL) system; B, control (ΔRL) system; ASM and BSM; ^{14}C in the methanol-water phase of each supernatant; ASC and BSC, ^{14}C in the chloroform phase of each supernatant.

lipid fractions. The silica gel in the area corresponding to each of these spots was scraped off and its radioactivity was measured in a liquid scintillation counter. The counts obtained were plotted against time as shown in Fig. 5.

A little PE appeared at the beginning of the immune bactericidal reaction in the lipid fraction of the supernatant, but later it did not increase much. FFA and LPE appeared a little later, and increased during the reaction.

3. *Free fatty acids (FFA) in the supernatant*

As shown in Figs. 2 and 5, more FFA than lyso-compounds appeared in the lipid fraction of the supernatants from the bactericidal system. The amount of lyso-compounds should not be less than FFA if both were derived from phospholipids. Some lyso-compounds might have been lost in the methanol-water phase or in the interphase during the extraction, or they might have been degraded to, e.g., glycerophosphorylethanolamine (GPE) or further during the bactericidal reaction. However, no GPE could be detected in the reaction supernatant or precipitate. It is possible that FFA could also be derived from the lipid A fraction of the lipopolysaccharide (LPS) complex of the bacterial surface. To study this possibility, the spot of FFA on the TLC sheet of the reaction supernatant was scraped off and extracted. It was chromatographed to separate β-hydroxy-fatty acid from usual fatty acids by the methods of Braun and Rehn (1969) and Braun et al. (1970), since β-hydroxymyristic acid is known to be present only in lipid A in Gram-negative bacteria (Lüderiz et al., 1966).

As shown in Fig. 6, the FFA fraction from the supernatant of RL-treated sensitized bacteria contained little or no β-hydroxy acid. Therefore, it is obvious that none of the FFA fraction was derived from lipid A of the surface LPS complex.

DISCUSSION

Phospholipids or fragments containing phospholipid are released from complement-treated bacteria into the surrounding medium during the immune bactericidal reaction. In the early stage of this work, as shown in Table 1 and Fig. 1, the reaction mixtures were centrifuged at 65,000 *g* for 30 min to avoid contamination of the supernatant with large-sized particules (Inoue, 1971). Later it was found that no more isotope was present in the supernatant when the reaction mixture, either with or without lysozyme, was centrifuged at only 2,000 *g* for 10 min. However, after EDTA-sucrose-treatment or osmotic shock 1.5 to 4 fold isotope was found in the supernatant when

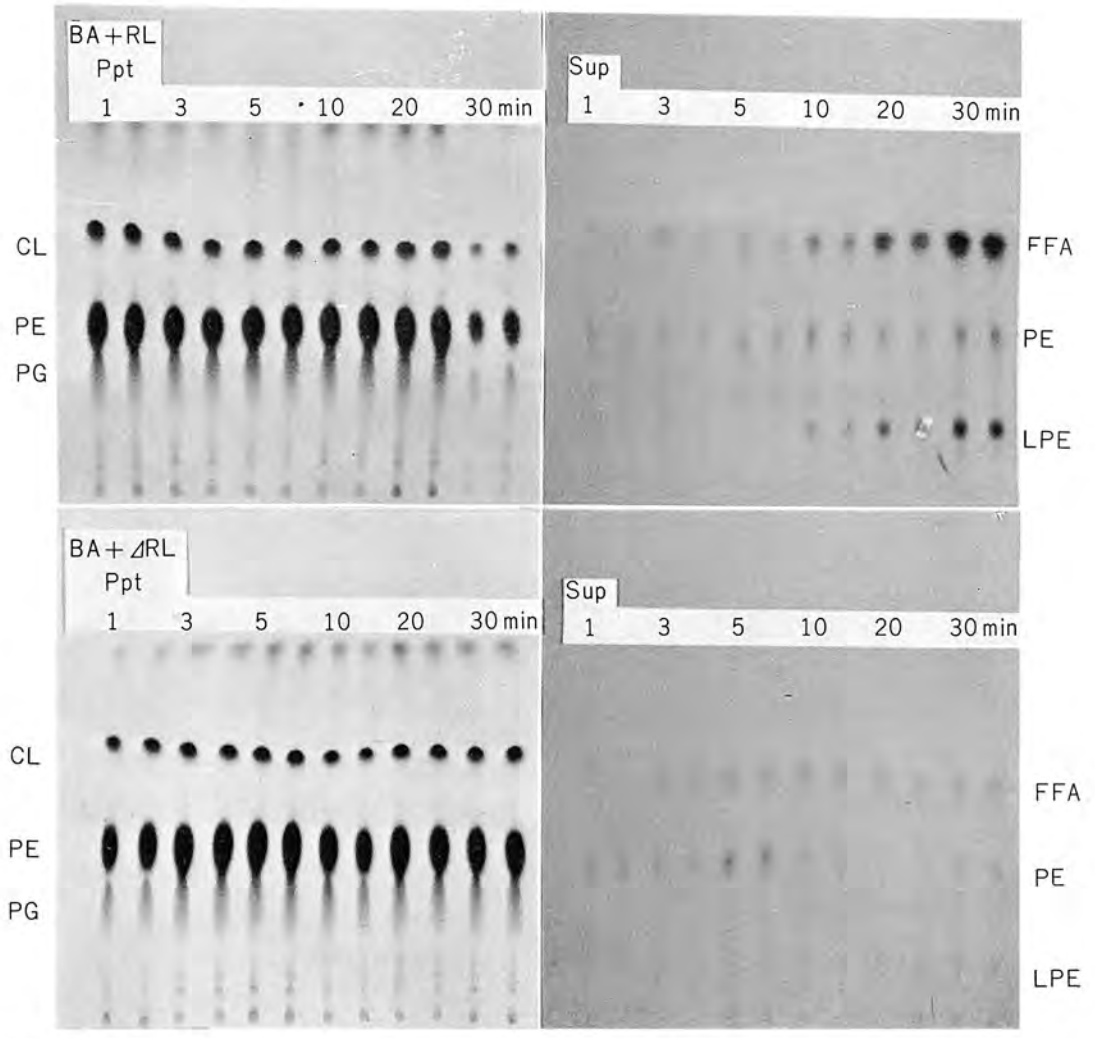


FIGURE 4. *Autoradiograms of thin layer chromatograms of lipid fractions from precipitates and supernatants from the immune bactericidal and control systems.*

Upper, immune bactericidal (BA+RL) system; bottom, control (BA+ΔRL) system; left, precipitate; right, supernatant. The figures indicate the time (minutes) when the aliquot was taken.

Chromatography was performed on sulica gel H plates on glass with chloroform-methanol-water (65: 25: 4, by vol.).

See legend of Fig. 1 for abbreviations indicating the position of spots.

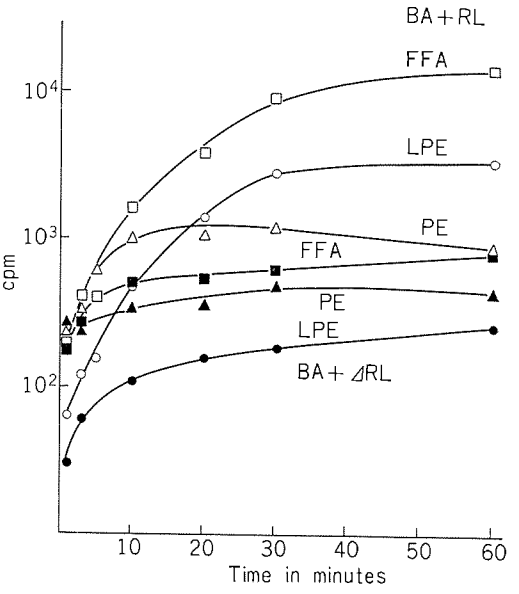
bacteria were centrifuged at the lower speed. Therefore, the fragments released from complement-treated bacteria seem to be small and fairly uniform in size, while those released from bacteria after EDTA-sucrose-treatment or osmotic shock may vary greatly in size.

Almost from the beginning of immune bactericidal reaction, a little PE appeared in the lipid fraction of the supernatant, but it did not increase in amount thereafter. Later FFA and LPE appeared in this fraction and gradually increased in amount.

The FFA fraction released from complement-treated bacteria contained no β-hydro-

xymyristic acid, showing that lipid A of LPS is not degraded when the bacteria are damaged by complement.

Smith and Becker (1968) reported an increase of titratable acid and a decrease of serine or choline phospholipid in the reaction mixture during immune cytolysis of sheep erythrocytes. They suggested that the changes of titrable acid might be related to the action of



▶

FIGURE 5. *Course of release of lipids into the chloroform phase of the supernatants from immune bactericidal system.*

Silica gel in the areas corresponding to the spots appeared in autoradiograms of TLC from the supernatants shown in Fig. 4 was scraped off and counted. Open symbols for the immune bactericidal (RL) system. Closed symbols for the control (⊿RL) system.

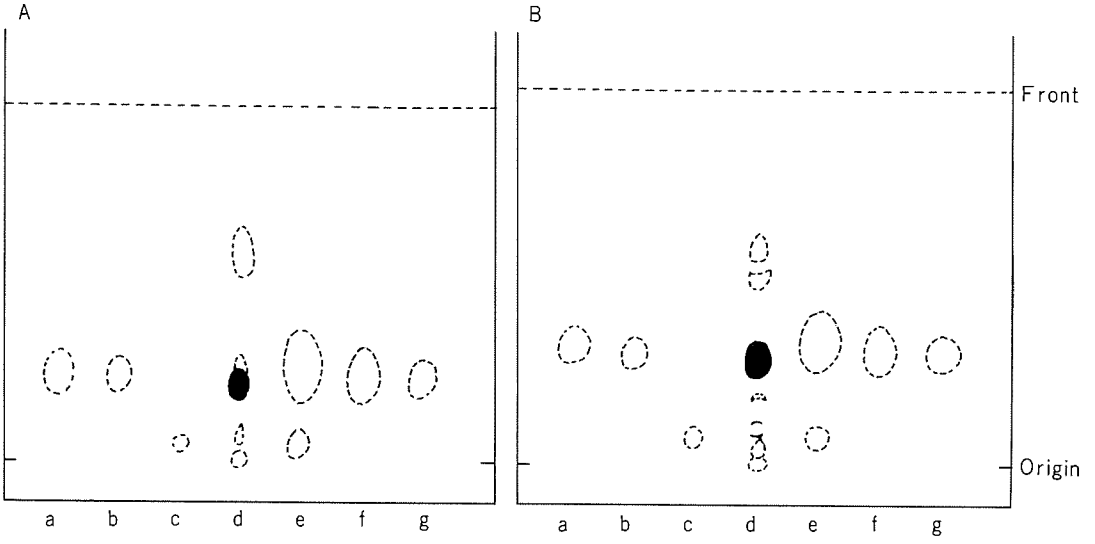


FIGURE 6. *Thin layer chromatogram of free fatty acid fractions obtained from the supernatant of the immune bactericidal system.*

The lipid fraction from the immune bactericidal (RL) system was chromatographed on a silica gel thin layer. Silica gel in the area corresponding to the spot of free fatty acid on the autoradiogram was scraped off and extracted. The extract (d) was rechromatographed with authentic samples of fatty acids on aluminum sheet silica gel (Merck 5553) with heptane-ethylacetate (4: 1, v/v) (A) or petroleum ether (60–70C)-ether-acetic acid (70: 30: 2, by vol.) (B). (Braun et al., 1969, 1970). After making autoradiograms, the sheets was sprayed with iodine solution in chloroform to visualize spots. Dotted lines indicate iodine-positive spots. Black areas indicate spots appearing on autoradiograms. a, stearic acid; b, myristic acid; c, β-hydroxymyristic acid; d, free fatty acid fraction from the lipid fraction of the supernatant of the immune bactericidal system; e, mixture of stearic, myristic, oleic, linolic and β-hydroxymyristic acids; f, oleic acid; g, linolic acid.

late-acting complement components and not of intracellular or serum enzymes following the action of complement on the erythrocyte membrane. However, our results show that PE appears first in the surrounding medium, while its degradation products appear later. Moreover, we found that treatment of sheep erythrocytes with purified phospholipase A from "Habu" (*Trimeresurus flavoviridis*) venom did not cause hemolysis, even after most of the membrane phospholipids had been converted into lyso-compounds, which remained in the membrane (unpublished data).

We also found that a phospholipase A-deficient mutant of *E. coli* B releases PE, but little or no FFA or LPE, into the lipid fraction of the supernatant when treated with antibody plus complement as shown in the following paper (Inoue et al., 1974).

Therefore, it seems likely that aggregates of late-acting complement components produce the lesions on or in the outer membrane of the bacteria, and cause the release of PE or fragments containing PE, which have been rendered susceptible to bacterial phospholipase A, from the outer membrane of sensitized bacteria into the surrounding medium.

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