Degradation of macromolecules such as DNA, RNA, protein, and phospholipid in carotovoricin Er-treated cells was examined. No significant decrease in acid-insoluble radioactivity of DNA, RNA, or protein which were labeled with \(^{3}H\)-thymine, \(^{3}H\)-uracil, and \(^{3}H\)-phenylalanine, respectively, could be detected in the carotovoricin Er-treated cells. On the other hand, marked degradation of phospholipids labeled with \(^{2-3}H\)-glycerol was observed in the cells exposed to the bacteriocin. There was a similar decrease in radioactivity of both \(^{3}H\) and \(^{32}P\) in the lipid fraction double-labeled with \(^{2-3}H\)-glycerol and \(^{32}PO_{4}^{2-}\). This decrease in radioactivity of phospholipids was explained by the release of fatty acid moieties from phospholipids. These results indicate that phospholipid degradation in carotovoricin Er-treated cells is caused by the action of phospholipase A. The amount of fatty acids released from phospholipids was proportional to the number of cells killed by the bacteriocin, suggesting that phospholipase A activity involved in phospholipid degradation is located in the sensitive cells, not in the bacteriocin.

Carotovoricin Er, a bacteriocin from \textit{Erwinia carotovora} strain Er, is a high molecular weight bacteriocin which consists of a contractible sheath, a core, and fibers, and resembles the tail part of a bacteriophage (1). In a previous paper (2), we reported that carotovoricin Er induced rapid and extensive cellular lysis of sensitive strains. We studied the degradation of macromolecules during the lysis caused by the bacteriocin and found that specific and marked degradation of phospholipids occurred. We describe here the nature of the phospholipid degradation, and also discuss a possible enzyme involved in this degradation.
MATERIALS AND METHODS

Bacterial strain. *Erwinia carotovora* Er (AMS 6082) was used as a bacteriocinogenic strain and *E. carotovora* 645Ar as a strain sensitive to carotovoricin Er. A thymine-requiring mutant, *E. carotovora* 645ArT, was induced from *E. carotovora* 645Ar with N-methyl-N'-nitro-N-nitrosoguanidine by the method of Adelberg et al. (3) and selected by the method of Stancy and Simon (4). *E. carotovora* 645ArT required 50 μg of thymine per ml in the medium to reach the same growth level as that of the parent strain.

Medium. The organisms were usually grown in M9 minimal medium supplemented with 0.2% each of glucose and casein acid hydrolysate as described previously (2). Thymine (50 μg/ml) was supplied to the medium when it was required.

Preparation of carotovoricin Er. Carotovoricin Er was induced with mitomycin-C and purified and prepared for use as described previously (1, 2).

Labeling of cells. To label the cells, *E. carotovora* 645ArT was grown in M9 medium containing [6-3H] thymine (5 μCi/ml), [5-3H] uracil (0.25 μCi/ml), L-phenyl [2, 3-3H] alanine (0.2 μCi/ml) or [2-3H(N)] glycerol (1 μCi/ml) to a log-phase culture (about 3 x 10^8 cells/ml). When the cells were labeled with [5-3H] uracil, 10 μg of nonlabeled uracil per ml was added to the medium. Cells double-labeled with [2-3H] glycerol and 32P_4^- were prepared in the medium containing 4 μCi of [2-3H(N)] glycerol and 10 μCi of H_332P_4^- per ml. Acyl groups of phospholipids of *E. carotovora* 645ArT were labeled with [1-14C] acetate by growing the cells in M9 medium containing 0.4 μCi of [1-14C] acetate per ml. The bacteria were cultured at 30° in a reciprocal shaker. The labeled cells were centrifuged at 7,000 x g at room temperature for 7 min, washed with M9 buffer (2) and suspended in 0.05 M phosphate buffer containing 0.15 M NaCl, pH 7.2, at a cell concentration of 2.0–2.5 x 10^8 cells/ml.

Measurement of radioactivity in acid-insoluble fraction. A portion (0.1 or 0.2 ml) of the reaction mixture was withdrawn and mixed with the same volume of 10% ice-cold trichloroacetic acid (TCA). The precipitates formed were collected on a Millipore filter (HAWP, pore size 0.45 μm) and washed with 5% ice-cold TCA (10 ml). The filter was then placed in a counting vial and the precipitates were solubilized with 5% sodium dodecylsulfate (0.5 ml) by incubating at 37° overnight. Ten ml of nonion-toluene scintillation fluid (5) was added and radioactivity was counted in a Packard Tri-Carb 3385 liquid scintillation spectrometer.

Extraction and separation of lipids. Cellular lipids were extracted with chloroform by a modification of the method of Bligh and Dyer (6). Lipids extracted in chloroform were evaporated to dryness under reduced pressure and dissolved in a small volume of chloroform-methanol (2: 1, v/v). The sample was spotted on a precoated thin-layer plate (Silica gel 60, E. Merck, Darmstadt, West Germany). Development was carried out with two different solvent systems, chloroform-methanol-acetic acid (65: 25: 10, v/v) and petroleum ether-diethyl ether-formic acid (55: 40: 1.5, v/v). Each phospholipid and free fatty acid (FFA) was identi-
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Degradation of phospholipids by treatment with carotovoricin Er was studied by comparing its Rf value with those of standards after autoradiography using a Fuji X-ray film. The spots of phosphatidylethanolamine and FFA were confirmed by the ninhydrin reaction and exposure to iodine vapor, respectively. Oleic acid was used as FFA standard. Areas of radioactive spots as revealed by autoradiography were scraped off the thin-layer plate. Their radioactivity was measured by the liquid scintillation spectrometer in a toluene scintillation fluid.

Radioactive chemicals. [6-3H] thymine (21 Ci/m mole), [2-3H(N)] glycerol (143 mCi/m mole) were products of the Radiochemical Centre, Amersham, England. [5-3H] uracil (18.4 Ci/mmole) was purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo. H$_3$PO$_4$ (carrier free) was a product of the Japan Atomic Energy Research Institute, Tokyo.

RESULTS

Degradation of macromolecules accompanied by cell lysis induced by carotovoricin Er

Cellular macromolecules DNA, RNA, protein, and lipid of *E. carotovora* 645 ArT were labeled with 3H-thymine, 3H-uracil, 3H-phenylalanine and 2-3H-glycerol, respectively. These labeled cells were treated with carotovoricin Er in 0.05 M phosphate buffer, pH 7.2, containing 0.15 M NaCl. This treatment caused a marked lysis of the cells (2). As shown in Table 1, no significant decrease in radioactivity of DNA, RNA, or protein in the acid-insoluble fraction was observed, while radioactivity in the acid-insoluble fraction from 2-3H-glycerol-labeled cells decreased to about one-half during the 60-min incubation period. More than 95% of the radioactivity of 2-3H-glycerol-labeled cells was extracted with

<table>
<thead>
<tr>
<th>Macromolecules</th>
<th>Carotovoricin</th>
<th>TCA-insoluble 3H dpm at 60 min</th>
<th>TCA-insoluble 3H dpm at 0 min (%)</th>
<th>Survival (%)</th>
</tr>
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<td>DNA</td>
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<td></td>
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<td>55</td>
<td>7.8</td>
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</table>

Labeled cells of *E. carotovora* 645 ArT were suspended in 0.05 M phosphate buffer, pH 7.2, containing 0.15 M NaCl at a concentration of 2.5 x 10$^5$ cells/ml. The cell suspensions (1 ml each) were incubated at 30° for 5 min, and 0.1-ml quantities of bacteriocin solution (100 units/ml) or of 0.5% bovine serum albumin as a control were added to the suspensions. Radioactivity in the ice-cold TCA-insoluble fraction was measured. Viable cells were counted on nutrient agar plates and survival was expressed as percent of the survival of control cells.
chloroform, indicating specific labeling of lipids by 2-3H-glycerol. The number of viable cells in the suspensions to which the bacteriocins were added was 5.7-8.9% of those of the control cell suspensions as shown in the table. The loss of radioactivity of the acid-insoluble fraction from 3H-glycerol-labeled cells began immediately after addition of the bacteriocin, and was dependent on the incubation time and the amount of bacteriocin added (Fig. 1). Viable cells in the cell suspensions to which 2.5 and 10 units of the bacteriocin per ml were added were 33.8% and 1.7% of those of the control. These results show that lipid was degraded specifically and without lag after challenge by the bacteriocin.

Mode of degradation of membrane phospholipids in carotovoricin Er-treated cells

The major lipids of Gram-negative bacteria are known to be phospholipids which are located in the outer and inner membranes (7, 8). These phospholipids are composed of phosphatidylethanolamine (PE, 75-85%), phosphatidylglycerol (PG, 10-20%) and cardiolipin (CL, 5-15%) (9). The phospholipids of E. carotovora 645ArT were double-labeled with [2-3H(N)] glycerol and 32P04-. The labeled cells were treated with carotovoricin Er and the decrease in radioactivity in the lipid fraction of the cells was measured. As shown in Fig. 2, radioactivity of 3H
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and $^{32}$P in the lipid fraction of the bacteriocin-treated cells decreased at the same rate. At 20 min the viable cells of the reaction mixture to which the bacteriocin was added had decreased to 2.7% of that of the control mixture. The data in Fig. 2 suggest that degraded lipid materials resulting from carotovoricin Er treatment were phospholipids and that hydrolysis of the ester bond between phosphate and the glycerol backbone did not occur. When [1-$^{14}$C] acetate-labeled cells were challenged by carotovoricin Er, a marked release of the fatty acid moieties of phospholipids was observed, but no diglyceride was detected (Fig. 3). As shown in Fig. 4, the amount of free fatty acid released corresponded to the loss of radioactivity from phospholipids in bacteriocin-treated cells. Recovery of radioactivity in the free fatty acid and lipid fraction of carotovoricin Er-treated cells was usually more than 90% of that of control cells. The number of viable cells in the mixture containing the bacteriocin at 20 min was 9.0% of that in the control mixture.

Change of phospholipid composition in carotovoricin Er-treated cells

To determine the phospholipid species from which the fatty acid was liberated, [1-$^{14}$C] acetate-labeled cells of E. carotovora 645ArT were exposed to the
bacteriocin, and phospholipids were extracted and separated by thin-layer chromatography. The phospholipids were found to consist of PE, PG, and CL, in concentrations of 78.5%, 14.0%, and 7.5%, respectively, as determined by distribution of radioactivity. Figure 5 shows that, in carotovorin Er-treated cells, free fatty acids were released rapidly from PE and PG after addition of carotovorin Er, while only a very small change in CL was observed. Lysophospholipid accumulated as an intermediate compound and then decreased during further incubation (Fig. 5). Viability of the suspension to which carotovorin Er was added was 11.2% of that of the control suspension at 20 min.
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The amount of free fatty acids released from phospholipids was plotted against the bacteriocin concentration or the number of affected cells. As shown in Fig. 6, plots of the amount of released free fatty acids against the number of affected cells gave a linear relationship, suggesting that the enzyme involved in the degradation of phospholipids is located in the sensitive cells.

Effect of Mg$^{2+}$ on the release of fatty acids from phospholipids in carotovoricin Er-treated cells

The lysis of sensitive cells induced by the bacteriocin was repressed by Mg$^{2+}$ (2). We also observed a protective effect of Mg$^{2+}$ on the release of fatty acid from phospholipids in carotovoricin Er-treated cells. Mg$^{2+}$ (5 mM) partially protected the sensitive cells from the killing action of the bacteriocin. At 20 min, the viable cell counts of the mixtures treated with 4 units of carotovoricin Er per ml in the presence or absence of 5 mM Mg$^{2+}$ were 56% and 25% of those of the control mixture, respectively. As shown in Fig. 7, the rate of release of fatty acid from the cells treated with carotovoricin Er in the presence of 5 mM Mg$^{2+}$ decreased about 1/7 as much as the cells treated with the bacteriocin in the absence of Mg$^{2+}$.
We reported previously (2) that carotovoricin Er, a bacteriocin from Erwinia carotovora Er, caused prompt and extensive lysis of its sensitive cells. We have now studied the degradation of macromolecules accompanied by the lysis induced by the bacteriocin, and found that marked degradation of phospholipid occurred in the bacteriocin-treated cells. Major phospholipid classes of E. carotovora 645-ArT, a carotovoricin Er-sensitive strain, were PE, PG, and CL, and the content of each, determined by distribution of [1-14C] acetate, was 78.5%, 14.0%, and 7.5%, respectively. These values are comparable with those of Escherichia coli (9).

**DISCUSSION**

A suspension of cells labeled with [1-14C] acetate was prepared as described in the legend for Fig. 1. Five-tenth-ml quantities of the bacteriocin solution (40 units/ml) or of 0.5% bovine serum albumin was added to 2.5 ml of the cell suspensions, and the mixtures were incubated at 30°. Samples (0.3 ml) were withdrawn at various times, and lipids were extracted and separated on a thin-layer plate with chloroform-methanol-acetic acid (65: 25: 10, v/v). ○, lipids from control cells; ●, lipids from carotovoricin Er-treated cells. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; FFA, free fatty acid; LPL, lysophospholipid.

**Fig. 5.** Change of phospholipid composition in carotovoricin Er-treated cells.
Fig. 6. Relationship between the amount of released fatty acid and the bacteriocin concentration or affected cells.

A suspension of *E. carotovora* 645ArT labeled with [1-¹⁴C] acetate was prepared as described in the legend for Fig. 1. Mixtures (1 ml) containing 0.9 ml of the cell suspension, and 0 to 0.08 ml of the bacteriocin solution (100 units/ml) as indicated were incubated at 30°. At 60 min, ○, and 120 min, ●, 0.3-ml samples were withdrawn and lipids were extracted and separated by thin-layer chromatography with petroleum ether–diethyl ether–formic acid (55: 40: 1.5, v/v). The number of affected cells was determined by counting viable cells on nutrient agar.

Fig. 7. Protective effect of Mg²⁺ on phospholipid degradation in carotovoricin Er-treated cells.

A suspension of cells labeled with [1-¹⁴C] acetate was prepared as described in the legend for Fig. 1. The mixtures (1 ml) containing 0.8 ml of the cell suspension and 4 units of the bacteriocin per ml, with or without 5 mM MgCl₂, were incubated at 30°. Samples (0.2 ml) were withdrawn at various time intervals and lipids were extracted and separated. Viable cell counts of these mixtures at 20 min after challenge were determined on nutrient agar plates. ○, with carotovoricin and no MgCl₂; ●, with carotovoricin and 5 mM MgCl₂.
Degradation of phospholipids due to carotovoricin Er was caused by deacylation of fatty acid moieties, and no breakage of ester linkage between phosphate and glycerol skeleton and no formation of diglyceride nor phosphatidic acid occurred (Fig. 2 and Fig. 3). PE and PG were deacylated while the content of CL did not change significantly. As shown in Fig. 5, a small amount of lysophospholipid accumulated as an intermediate compound and then decreased during further incubation. This result suggests that deacylation of PE and PG was possibly catalyzed by phospholipase A, and this resulted first in the formation of free fatty acids and lysophospholipid, and the lysophospholipid thus formed might be further deacylated by a lysophospholipase. The observation that the amount of free fatty acid liberated was dependent on the number of cells affected by carotovoricin Er, rather than the concentration of the bacteriocin added, indicated that a phospholipase involved in the deacylation of PE and PG in the affected cells is located in the sensitive cells. We have characterized phospholipase A of \textit{E. carotovora} 645ArT to some extent and found that major phospholipase A activity of this organism is located in the membrane fraction, is specific for PE and PG, and is activated by detergents, methanol and Ca$^{2+}$. These characteristics are quite similar to those of dr-phospholipase A of \textit{E. coli} (10). By using a mutant of \textit{E. carotovora} 645ArT deficient in phospholipase A, we established that the deacylation of phospholipid caused by the bacteriocin was due to the action of phospholipase A (unpublished results). Degradation of phospholipid in colicin K- or E1-treated \textit{E. coli} has been reported (11, 12). In colicin K-treated \textit{E. coli} (12), the level of lysophosphatidylethanolamine increased because of the action of dr- and ds-phospholipase A (10, 13). The amount of lysophosphatidylethanolamine in the colicin K-treated cells, however, was less than 5% of the total phospholipids when the cells were treated with colicin K at a multiplicity of 10–30 at 37° for 1 hr (12). The deacylation of phospholipid has also been reported in cells infected by T4 and T4 ghosts (14, 15). BULLER \textit{et al.} (14) have reported that dr- and ds-phospholipase A were also involved in the formation of free fatty acids or lysophospholipid and that about 10% of the total phospholipid is deacylated during 30-min incubation at 37° after the attachment of T4 ghost to the host cells. In the case of free fatty acid formation caused by carotovoricin Er, more than 50% of free fatty acids were formed when the sensitive cells were treated with the bacteriocin at 30° for 60 min at a multiplicity of 3–4. Figure 7 shows that Mg$^{2+}$ repressed free fatty acid formation in the carotovoricin Er-treated cells. It is well known that divalent cations such as Mg$^{2+}$ and Ca$^{2+}$ play an important role in the maintenance of structural rigidity and the permeability barrier function of the cell envelope of Gram-negative bacteria (16, 17). We postulate that treatment with carotovoricin Er resulted in activation of membrane-bound phospholipase A and subsequently degradation of membrane phospholipids resulted.

We are grateful to the Kyowa Hakko Kogyo Co. for the generous supply of mitomycin-C and Miss A. Sato of this faculty for her help in the operation of the liquid scintillation spectrometer.
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This investigation was supported in part by Grant-in-Aid for Scientific Research (to H. T., 443026) from the Ministry of Education, Science, and Culture of Japan.

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