Leukocidin, Tetraethylammonium Ions, and the Membrane Acyl Phosphatases in Relation to the Leukocyte Potassium Pump

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ABSTRACT The response of the leukocyte to leukocidin and its relevance to excitable and secreting tissues are described. New data are presented on the leukocyte membrane phosphatases and the action of tetraethylammonium ion (TEA) on the leukocyte. The leukocyte surface membrane lacks a cation-sensitive ATPase but possesses a potassium- and ouabain-sensitive p-nitrophenyl phosphatase. The p-nitrophenyl phosphatase shows peak activity at three pH values and the pH dependence and potassium sensitivity depend on the state of the membranes. In the presence of magnesium, potassium can stimulate over the range pH 6 to 8. The relation of the leukocyte p-nitrophenyl phosphatase to electrolyte control in the leukocyte and to the properties of cation-sensitive phosphatases in other cells suggests that the leukocyte enzyme is a component of an electrogenic potassium pump. Leukocidin stimulates the leukocyte p-nitrophenyl phosphatase under all the conditions studied. The effect is specific and occurs under conditions that induce cytotoxic effects in the cell. It is concluded that the potassium pump is the site of action of leukocidin. TEA prevents the effects of leukocidin by inhibiting the action of leukocidin and not the responses of the cell to injury. TEA does not inhibit the p-nitrophenyl phosphatase nor prevent its stimulation by leukocidin. The enhancement of leukocidin by diisopropylphosphofluoridate (DFP) is briefly described. It is concluded that TEA acts in the opposite way to DFP and blocks the ion pathway activated by leukocidin in the leukocyte potassium pump.

INTRODUCTION

Leukocidin is an extracellular product of the Straphylococcus, consisting of two proteins, that kills the polymorphonuclear leukocytes of rabbit and man. The two proteins, called the F component and the S component of leukocidin, are globular, water soluble, have molecular weights of about 30,000, and have both been crystallized. They act synergistically; neither is toxic alone.

Among the effects that leukocidin produces in leukocytes are an increased
permeability to cations, secretion of the proteins in the cytoplasmic granules, and an intracellular accumulation of calcium in vesicles. These changes resemble those seen in excitable tissues during membrane depolarization or in secreting tissues following stimulation with hormones. Comparison of the effects of leukocidin, streptolysin O, and excess vitamin A on the leukocyte suggested that the specific characteristics of leukocidin action are the way in which the permeability is changed and the orthophosphate produced in the cytoplasm (1). This led Woodin and Wieneke (2) to suggest that leukocidin might produce a structural change in an ion pump. The action of leukocidin on the cation-sensitive phosphatases of the leukocyte membrane supported this speculation and suggested that the pump might be specific for potassium (3).

The first group experiments in this paper describes the properties of the cation-sensitive phosphatases of the leukocyte membrane and their modification by leukocidin. The second group of experiments describes the inhibition of the effect of leukocidin by tetraethylammonium ions (TEA). The inhibition has been studied by the methods used to demonstrate the enhancement of leukocidin action by diisopropylphosphofluoridate (DFP) (4) and to clarify the rationale of these experiments a brief account is given of the response of the leukocyte to leukocidin.

The two proteins that constitute leukocidin and that act synergistically upon the leukocyte cell membrane are simultaneously converted to an inactive form. It is probable that there is a reciprocal conformational change between leukocidin and lipid components of the membrane and that the inactivation of leukocidin involves contact with triphosphoinositide (2). In the isolated cell membrane leukocidin action is manifested as an increased acyl phosphatase activity. In the intact cell the action leads to an increased cation permeability and an accumulation of orthophosphate in the cytoplasm. Subsequent events are dependent on the presence of calcium. If this is present in the medium secretion of the proteins in the cytoplasmic granules is induced, calcium phosphate is deposited in vesicles derived from the empty granules, and a new orthophosphate-nucleotide exchange reaction is set up on the cell membrane. If calcium is not present in the medium these reactions do not occur but, if calcium is added, they are induced even after the excess leukocidin has been neutralized by antibody. In the leukocyte the reactions dependent upon calcium-coupling can be distinguished from those associated with excitation by leukocidin. Thus reagents that act exclusively on the calcium-dependent reactions can be identified by adding them to leukocidin-treated cells after the leukocidin has been neutralized by antibody but before calcium is added. Conversely, reagents that act on the excitation by leukocidin should only be effective when they are present at the same time as leukocidin and then should modify all the responses of the cell. Reagents specific to leukocidin
can also be identified from their effect on the response of the cell to streptolysin O or vitamin A.

In screening compounds that affect the leukocidin-treated cell those that act on the individual responses of the cell after leukocidin has been neutralized are first eliminated. Compounds that affect all the responses of the leukocyte when leukocidin is present (but not those induced by streptolysin O) can be claimed to have sites of action identical with leukocidin. If such compounds, in addition, affect both the response of the isolated leukocyte cell membrane and the inactivation of leukocidin by the membrane, it can be claimed that the compounds and leukocidin interact with the same molecules. No such compound has been found. DFP has been found to act at the same site as leukocidin (4) and the present paper shows that TEA also does.

Earlier work (1, 5) has shown that the efflux of potassium in the leukocidin-treated leukocyte is not due to inhibition of active transport. Leukocidin activates a pathway facilitating ion movements. The present paper provides further evidence that this pathway is situated in the potassium pump of the cell membrane. It is suggested that DFP enhances and TEA inhibits the action of leukocidin by respectively stabilizing and blocking the ion pathway.

**METHODS**

**Materials**

Leukocytes were obtained from peritoneal exudates of rabbits (5). The F component and the S component of leukocidin were the crystalline products obtained after purification of culture filtrates of the V8 strain of *Staphylococcus aureus* (6). In most of the experiments described here both components were present in equal amounts and the statement "x micrograms of leukocidin" will mean "x micrograms of each component of leukocidin." Solutions of the crystalline material (1 mg/ml) were prepared in 0.145 M NaCl solution and serially diluted in 0.145 M NaCl solution containing 1 mg/ml of bovine serum albumin. Streptolysin O was obtained from the Wellcome Research Laboratories, Beckenham, England. It was serially diluted in 0.145 M NaCl solution containing 1 mg/ml of bovine serum albumin and 0.1 mM cysteine, adjusted to pH 7.2 with dilute NaOH. The units of streptolysin O activity used in this paper are those specified by the manufacturer. Diisopropylphosphofluoridate (DFP) was kindly provided by the Chemical Defence Research Establishment, Porton, England. The phosphonic acid ester was a gift from Dr. E. Becker, Walter Reed Institute for Medical Research, Washington, D. C. Leukocyte surface and cytoplasmic membranes were obtained from 10^10 leukocytes. The cell suspensions in ascitic fluid were treated with 0.25 M Na-EDTA solution, pH 7.2, to give a final concentration of 2 mM and centrifuged at 3000 g for 5 min. The cell pellets were washed twice with 20 ml of 11.6% sucrose, 1 mM Na-EDTA solution, pH 7.2, by centrifuging in the same way and then homogenized for 2 min in a Potter-Elvehjem homogenizer. The homogenate was diluted to 50 ml with 11.6% sucrose, 1 mM Na-EDTA solution, pH 7.2, and 6 ml put on top of a discontinuous density gradient.
consisting of layers (8 ml each) of 30, 40, and 50% sucrose solution, all containing 1 mM Na-EDTA and centrifuged at 30,000 rpm for 1 hr in the No. 30 rotor of the Spinco ultracentrifuge, model L. The material between the 30 and 40% sucrose solutions is referred to as the leukocyte surface membrane fraction. The material between the 40 and the 50% sucrose solutions is referred to as the leukocyte cytoplasmic membrane fractions. The identification of the surface membrane fraction is based on its inactivating leukocidin in the same way as leukocyte suspensions (7).

The Membrane Phosphatases

In order to increase the cation sensitivity of the leukocyte surface and cytoplasmic membranes they were treated in various ways: (a) NaI-treated membranes were made by the method of Israel and Titus (8); (b) treatment with dilute Tris buffer was done by washing the membranes with 12 volumes of 1 mM Tris buffer, pH 7.2, by centrifuging at 20,000 g at 0°C; (c) aged membranes were those that had stood at 2-4°C for 2 days. In order to determine the pH-activity relationship Tris-acetate buffers were used. The final molarity (0.1 M) refers to the Tris concentration and the recorded pH to that of the 0.28 M stock solutions of the buffers measured at room temperature. As the properties of leukocidin are modified at low ionic strength, the effect of leukocidin and streptolysin O on the membrane phosphatases was investigated in 0.1 M NaCl, 0.05 M Tris buffer, pH 7.2. Concentrated membrane suspensions (4-8 mg protein/ml) were used and then diluted 20- to 40-fold before determining the phosphatase activity. The hydrolysis of 2-glycerophosphate or adenosine-5-monophosphate was determined from the orthophosphate produced after 1 hr at pH 5 or 2 hr at pH 7.5 from solutions containing 5 mM substrate and 0.1 M Tris-acetate, pH 5, or Tris-chloride, pH 7.5, buffers, and 0.5 mg membrane protein/ml. The ATPase was determined as described by Woodin and Wieneke (3). In the p-nitrophenyl phosphatase assay special precautions were taken to prevent surface denaturation. Plastic, nonwettable tubes, 3 mm in diameter, were used and special pipettes constructed to deliver reagents to the bottom of the tubes. In the assay, the tubes were immersed in ice and 10 μl of water or solutions of magnesium, potassium, or ouabain added. The membrane preparation in 40 μl of 11.6% sucrose solution was added and then 50 μl of 10 mM p-nitrophenyl phosphate in 0.2 M Tris buffer of the appropriate pH. The mixtures in the tubes were mixed without froth formation and the reaction started by incubation at 37°C. After 20 min incubation 0.4 ml of 0.2 M NaOH was added and the p-nitrophenol produced determined from the absorbance at 410 μm. All measurements were done in duplicate and these never differed by more than 3%. In the "blank" determinations the membranes were added after the 0.2 M NaOH at the end of the incubation.

The Effect of TEA on the Leukocidin-Treated Leukocyte

The inhibition of leukocidin by TEA was demonstrated using the procedures employed to study the enhancement of leukocidin by phosphonates and full details are given by Woodin and Wieneke (4). The general method was to wash the leukocytes in the appropriate physiological salt solution and incubate them (5 X 10⁷ cells/ml) for 10 min at 37°C in this or in the corresponding solution in which part of the NaCl
had been replaced by a quaternary ammonium chloride. Serially diluted leukocidin was added and after a further 10 min at 37°C the secretion of β-glucuronidase, production of orthophosphate, intracellular accumulation of calcium, or cell swelling were determined by analyzing the cell pellet or supernatant. The chemical procedures used previously (4) could be applied to solutions containing TEA except the determination of orthophosphate by the Berenblum and Chain method (9). Here TEA caused precipitation of some of the phosphomolybdate in the isobutanol layer and, moreover, if the extract of the cells in trichloroacetic acid was not completely free from proteins some of the precipitated phosphomolybdate accumulated at the interface between the isobutanol and aqueous layers. Consequently, to determine the orthophosphate produced by leukocytes in the presence of TEA, the cell suspensions were precipitated with trichloroacetic acid (5% w/v final concentration) containing ³²P orthophosphate (5-10 × 10³ cpm per sample). After centrifugation, the orthophosphate in the supernatant was precipitated as the calcium salt (10) and the Berenblum and Chain (9) method applied to the precipitated material. The recovery of the orthophosphate was calculated from the radioactivity found in the solution of the reduced phosphomolybdate in isobutanol. Potassium leakage was determined by a refinement of the method used earlier (4). When leukocytes are washed at room temperature they lose potassium but on subsequent incubation at 37°C this is regained. It was found that 15-20 min incubation at 37°C is sufficient to bring the internal potassium concentration into a steady state and in studying the effect of TEA on potassium loss, cells were incubated in Hanks BSS (containing glucose and bovine serum albumin, 1 mg/ml of each) or the corresponding medium in which TEA-Cl replaced some of the NaCl, for 20 min at 37°C before adding the toxin. After 10 min incubation with the toxin the cell suspensions were centrifuged at 37°C and the supernatant removed. The cell pellets were extracted with boiling water and the potassium content determined by flame photometry. The wet weight of the pellet from 10⁸ cells is about 100 mg and as the potassium concentration of Hanks BSS is only 6.0 mM it was considered unnecessary to make allowances for potassium in the extracellular volume of the pellet. The effect of TEA on potassium accumulation by cooled cells was determined by the procedure of Elsbach and Schwartz (11) except that the cells were rewarmed in Hanks BSS containing 1 mg/ml bovine serum albumin and 1 mg/ml of glucose and not in ascitic fluid.

RESULTS

The Leukocyte Membrane Phosphatases

A. Properties of the Potassium-Sensitive Phosphatase

Preliminary experiments by Woodin and Wieneke (3) have shown that the leukocyte membrane phosphatases differ from those of other cell membranes. Thus the ATPase is not sensitive to sodium, potassium, or ouabain. The acyl phosphatase, on the other hand, with p-nitrophenyl phosphate, acetyl phosphate, or carbamyl phosphate as substrate, is stimulated by potassium and this stimulation is abolished by ouabain. The relationship of the cation-sensitive
acetylphosphatase to the cation-sensitive ATPase is not clear for any cell and so, in addition to their relevance to the mode of action of leukocidin and the mechanism of electrolyte control in the leukocyte, the properties of the leukocyte acetylphosphatase may have a general interest.

The p-nitrophenyl phosphatase activity of fresh membrane preparations was stimulated only 5-10% by magnesium but after ageing or treatment with Tris buffer or sodium iodide the effect of magnesium was more marked. Stimulation by potassium was only found with preparations that were sensitive to magnesium and then only in the presence of magnesium (Table I). With seven preparations of sodium iodide-treated membranes tested at pH 7.2 the stimulation due to 5 mM magnesium alone was in the range 32 to 55% (mean 41%) and that due to 5 mM magnesium and 10 mM potassium together was in the range 105 to 130% (mean 120%). The stimulation by potassium was reduced by ouabain in a competitive fashion (Table II). With other preparations of sodium iodide-treated membranes the stimulation due to 10 mM

### Table I

**Effect of Potassium and Magnesium on p-Nitrophenyl Phosphatase of Leukocyte Surface Membranes**

| Membrane treatment                        | No addition | 10 mM K+ | 5 mM Mg++ | 5 mM Mg++ |
|-------------------------------------------|-------------|----------|-----------|-----------|
| Aged at 0°C for 2 days                    | 0.85        | 0.78     | 0.94      | 1.10      |
| Washed with 1 mM Tris buffer, pH 7        | 0.75        | 0.70     | 0.9       | 1.10      |
| Treated with sodium iodide solution       | 0.34        | 0.34     | 0.46      | 0.75      |

Details of the treatment of the membranes are given in the Methods section.

### Table II

**Competitive Effect of Potassium and Ouabain on the p-Nitrophenyl Phosphatase Activity of Leukocyte Surface Membranes Treated with Sodium Iodide Solution**

| K concentration | No ouabain | 5 × 10^-4 m ouabain | 10^-4 m ouabain | 0.5 × 10^-4 m ouabain |
|-----------------|------------|---------------------|-----------------|---------------------|
| mM              | % potassium-free system | % potassium-free system | % potassium-free system | % potassium-free system |
| 1               | 17         | 0                   | 0               | 0                   |
| 2               | 26         | 0                   | 7               | 7                   |
| 5               | 31         | 7                   | 14              | 19                  |
| 10              | 37         | 7                   | 19              | 27                  |

In the absence of potassium and ouabain the p-nitrophenol produced was 0.6 µmole/hr per mg protein.
potassium has been abolished completely (three preparations) or reduced (three preparations) to less than a fifth of its original value by $5 \times 10^{-4}$ M ouabain.

The effect of pH on the p-nitrophenyl phosphatase activity of the membrane was also dependent on the state of the membranes. Immediately after preparation of the membranes the regions of optimum activity could be distinguished, with buffers at pH 5.0–5.5, 6.0–6.5, and 7.0–7.4 (Fig. 1). The mean pH values of the maxima in these regions, measured in the reaction mixtures at 37°C, will be pH 5.4, 6.5, and 7.35, respectively. Although the pH-activity profiles do not show discrete peaks the three regions have been observed in the seven preparations investigated and it is clear that the points in Figs. 1–3 cannot be fitted by symmetrical smooth curves. In aged membranes or those treated with dilute Tris buffer or sodium iodide solution there was a general decrease in activity that was most marked at neutral pH (Figs. 1 and 2). In treated preparations stimulation of the p-nitrophenyl phosphatase activity by magnesium was observed at all pH values above 6.0. When magnesium was present potassium produced a constant increment in the activity between pH 6 and 8 although the percentage stimulation is greatest at pH 7–8 (Fig. 2). There is no doubt that the stimulation by potassium is not restricted to p-nitrophenyl phosphate hydrolysis at neutral pH.

The cytoplasmic membrane fraction also had p-nitrophenyl phosphatase activity. The specific activity of membranes tested at pH 7.2 immediately after preparation was about 50% of that of the surface membranes. After treatment with dilute Tris buffer or sodium iodide solution the cytoplasmic membranes became sensitive to potassium in the presence of magnesium and
the stimulation due to 10 mM potassium was either reduced or abolished by $5 \times 10^{-4} \text{M ouabain}$. The percentage stimulation by magnesium and potassium was similar to that found with the surface membranes.

Fig. 3 shows the pH-activity relationship for cell surface and cytoplasmic membranes prepared at the same time. Both preparations have three maxima but the ratio of activities of the membrane preparations is not a constant function of the pH. It is unlikely that the p-nitrophenyl phosphatase activity of the cytoplasmic membranes is due to contamination with the surface membranes. Further evidence for this is provided by the effect of leukocidin described below.
The surface and cytoplasmic membranes also hydrolyzed β-glycerophosphate and adenosine-5-monophosphate (Table III). The hydrolysis of these substrates was not changed in the presence of 5 mM magnesium alone, or together with 10 mM potassium, nor was it changed by treating the membranes with sodium iodide. The hydrolysis of p-nitrophenyl phosphate at both pH 5 and pH 7.2 was reduced by the sodium iodide treatment and it is possible that at both pH values the hydrolysis is due to a specific p-nitrophenyl phosphatase. The action of leukocidin (see below) is also consistent with this conclusion.

| Membrane origin and treatment                          | at pH 5 | at pH 7.5 |
|---------------------------------------------------------|---------|-----------|
|                                                         | Glycero-| AMP | PNP | Glycero-| AMP | PNP |
|                                                         | phosphate |     |    | phosphate |     |    |
| Normal surface membranes                                | 0.15    | 0.090| 6.4 | 0.020    | 0.06 | 1.5 |
| Surface membranes after NaI treatment                   | 0.15    | 0.085| 1.8 | 0.025    | 0.06 | 0.32|
| Normal cytoplasmic membranes                            | 0.17    | 0.090| 3.4 | 0.031    | 0.075| 0.90 |
| Cytoplasmic membranes after NaI treatment               | 0.18    | 0.095| 0.3 | 0.035    | 0.07 | 0.25 |

The hydrolysis conditions are given in the Methods section. No Mg⁺ was present.

B. INHIBITION OF THE P-NITROPHENYL PHOSPHATASE AND THE NUCLEOSIDE TRIPHOSPHATASE

The p-nitrophenyl phosphatase of the surface membrane was not inhibited by 80 mM TEA, 5 mM DFP, 0.4 mM 5-chloropentyl-ethyl-p-nitrophenyl phosphate, 1 mM iodoacetamide, or 20 mM sodium fluoride. The first three of these substances modify the response of the leukocyte to leukocidin, and their failure to affect the p-nitrophenyl phosphatase is consistent with the view that their action is on the ion pathway activated by leukocidin (see below). N-Ethylmaleimide (1 mM) inhibited the p-nitrophenyl phosphatase in the presence and the absence of potassium. Calcium activated the enzyme to the same extent as magnesium but further activation by potassium was not found in the presence of calcium. The p-nitrophenyl phosphatase was also inhibited by nucleoside triphosphates (Fig. 4). It is of interest that this should occur in the leukocyte membrane for it has been found with other tissues that, unlike the leukocyte, possess a cation-sensitive ATPase (13). The phenomenon has even been taken as evidence that the cation-sensitive ATPase and the potassium-sensitive p-nitrophenyl phosphatase are not entirely separate (14).
The failure of numerous treatments to induce cation sensitivity in the leukocyte membrane ATPase was recorded earlier (3). It has also been found that the addition of the heated, soluble fraction from rabbit heart also failed to induce cation sensitivity. This fraction has been found to activate the cation-sensitive ATPase from rabbit muscle and dog parotid gland (15). The leukocyte ATPase was not inhibited by pretreatment with 5 mM DFP for 90 min. Hokin and Yoda (16) found that this treatment inhibited the cation-sensitive ATPase of kidney microsomes.

![Figure 4](image.png)

**Figure 4.** Inhibition of the p-nitrophenyl phosphatase activity of leukocyte surface membranes by nucleoside triphosphates. The assay mixtures contained 5 mM p-nitrophenyl phosphate and 5 mM Mg++.  

### C. The Action of Leukocidin on the p-Nitrophenyl Phosphatase

Woodin and Wieneke (3) described the stimulation by leukocidin of the p-nitrophenyl phosphatase of a leukocyte surface membrane preparation. In subsequent determinations of the p-nitrophenyl phosphatase activity at pH 7.2, 10 preparations of surface membranes showed 47–110% stimulation after leukocidin treatment (average 69%). In no case has a membrane preparation failed to respond to leukocidin and, in all cases tested, the stimulation has been found in the presence and absence of potassium. The treatment with dilute Tris buffer or sodium iodide solution was applied to three of these membrane preparations. The products showed 40–60% stimulation of the p-nitrophenyl phosphatase activity after leukocidin treatment. The stimulated activity in the leukocidin-treated membranes is restricted to the p-nitrophenyl phosphatase. The rate of hydrolysis of glycerophosphate or adenosine-5-monophosphate is not increased.

The action of leukocidin on the membrane is manifested as a stimulated p-nitrophenyl phosphatase activity over a wide pH range. With surface membrane preparations the stimulation showed peaks at about pH 6.0 and at pH 7.0–7.4. Cytoplasmic membranes also had an enhanced p-nitrophenyl phosphatase activity after leukocidin treatment but only a single peak of stimulated activity at about pH 6.5 was found (Fig. 5). This difference is of interest for
Wieneke and Woodin (7) found that the surface and cytoplasmic membranes differ in their ability to inactivate leukocidin. The different response of the surface and cytoplasmic membranes to leukocidin might be taken as evidence that leukocidin induces its effect upon p-nitrophenyl phosphatase indirectly, through an action on the neighboring molecules. There is good evidence that phospholipids are involved in the interaction of leukocidin with the leukocyte membrane (1, 12).

The conditions under which leukocidin stimulates the p-nitrophenyl phosphatases are those that induce the cytotoxic effects. Table IV shows that the stimulation is synergistic between the two components of leukocidin. The small effect found with each component alone can result from the two components not being completely free from each other. Both components must be present together; if one component of leukocidin is added to the membranes and washed away before adding the other component, no stimulation is found. Table V shows that maximum stimulation is found with 0.5–1.0 μg leukocidin/

![Figure 5](image)

**FIGURE 5.** The stimulation of the p-nitrophenyl phosphatase of surface and cytoplasmic membranes by leukocidin. Surface and cytoplasmic membranes were treated with leukocidin (2 μg of leukocidin/mg membrane protein) at pH 7.2 and then the p-nitrophenyl phosphatase activity determined at different pH values. The percentage increment of the activity produced by leukocidin is plotted for surface membranes (open circles) and cytoplasmic membranes (solid circles).

| Leukocidin present                  | p-Nitrophenol produced |
|------------------------------------|------------------------|
|                                    | μmoles/hr per mg of protein |
|                                    | No K present | 10 mM K present |
| 0                                  | 0.30         | 0.38          |
| F component only (15 μg/ml)        | 0.34         | 0.42          |
| S component only (15 μg/ml)        | 0.31         | 0.44          |
| F and S components present (7.5 μg/ml of each) | 0.56        | 0.75          |
| F and S components present (15 μg/ml of each) | 0.58        | 0.75          |

Tris-treated leukocyte surface membranes (7.5 mg protein/ml) were treated with one or both of the components of leukocidin and then diluted to 0.1 mg protein/ml before determining the p-nitrophenyl phosphatase activity.
Leukoidin, TEA, and Membrane Phosphatases

mg membrane protein; a mass ratio that leads to optimum inactivation of leukocidin by the membranes.

Some of the nonspecific responses of the leukocyte to injury by leukocidin are also brought about by streptolysin O. Table V shows that streptolysin O does not stimulate the \( p \)-nitrophenyl phosphatase but at high concentration inhibits. It is not known whether this inhibition is due to streptolysin O itself or to contaminating lipases. Bovine serum albumin, unfractionated horse serum, or a pepsin refined anti-serum to Staphylococcus aureus at 20 times the concentration used with leukocidin failed to alter the activity of the \( p \)-nitrophenyl phosphatase.

### Table V

| Leukocidin concentration | Relative activity of treated membrane | Streptolysin O concentration | Relative activity of treated membrane |
|--------------------------|--------------------------------------|-----------------------------|--------------------------------------|
| \( \mu \text{g/mg membrane protein} \) | \( \text{unit/mg membrane protein} \) | \( \text{unit/mg membrane protein} \) | \( \text{unit/mg membrane protein} \) |
| 0.12                     | 1.08                                 | 0.1                         | 1.05                                 |
| 0.25                     | 1.32                                 | 0.5                         | 1.02                                 |
| 0.50                     | 1.45                                 | 1.0                         | 1.00                                 |
| 1.0                      | 1.85                                 | 4.0                         | 0.75                                 |
| 2.0                      | 1.90                                 | 8.0                         | 0.60                                 |

Normal leukocyte membranes were treated with the amount of leukocidin or streptolysin O indicated and after dilution the \( p \)-nitrophenyl phosphatase activity was determined. The relative activity is the ratio of the activity of the treated membranes to that of the controls.

The Action of TEA on the Leukocyte

A. Inhibition of the Effect of Leukocidin on the Leukocyte

Table VI shows that 80 mM TEA inhibits the secretion of \( \beta \)-glucuronidase, accumulation of calcium, production of orthophosphate, and cell swelling when it is added to leukocytes before leukocidin. The effect is competitive and the inhibition by TEA is smaller in the presence of high leukocidin concentrations. The highest concentration of leukocidin used in the experiments recorded in Table VI is \( 6 \times 10^{-5} \text{ M} \) and the failure of \( 8 \times 10^{-3} \text{ M} \) TEA to inhibit some effects is evidence that TEA does not act directly on leukocidin. The inhibition of the effect of leukocidin is not reversed by prolonging the incubation. Fig. 6 shows the time course of calcium accumulation, for example. TEA did not inhibit the response of the leukocyte if it was added after leukocidin had been neutralized with antibody. If it was added to leukocidin-treated leukocytes prepared in the absence of calcium it did not inhibit the secretion of \( \beta \)-glucuronidase when calcium was subsequently added. Similarly
if it was added to leukocidin-treated leukocytes it did not decrease the rate at
which orthophosphate accumulated. Normal leukocytes treated with 80 mM
TEA and washed had a sensitivity similar to that of leukocytes maintained in
Hanks medium. It thus appeared that TEA was inhibiting the action of leuko-

Table VI
INHIBITION OF THE CYTOTOXIC EFFECTS
OF LEUKOCIDIN BY TEA

| Leukocidin concentration | β-Glucuronidase secreted | Calcium accumulated | Orthophosphate produced | Wet weight cell pellet |
|--------------------------|--------------------------|---------------------|------------------------|-----------------------|
|                          | NaCl                     | TEA-Cl              | NaCl                   | TEA-Cl                |
|                          | µg/10⁶ cells              | units/10⁶ cells     | µmole/10⁶ cells         | µg/10⁶ cells          | mg/10⁶ cells          |
| 0.05                     | 2                        | 0                   | 7.2                    | 1.2                   | 82                   | 78                   |
| 0.1                      | 2                        | 0                   | 10                     | 2.4                   | 1.5                  | 0.5                  | 84                   | 77                   |
| 0.2                      | 4                        | 2                   | 30                     | 8.5                   | 2.3                  | 0.7                  | 89                   | 78                   |
| 0.4                      | 16                       | 2                   | 53                     | 28                    | 4.0                  | 2.0                  | 98                   | 80                   |
| 0.8                      | 28                       | 10                  | 80                     | 64                    | 6.3                  | 3.0                  | 112                  | 92                   |
| 1.6                      | 56                       | 20                  | 85                     | 82                    | 8.8                  | 5.4                  | 160                  | 130                  |

Leukocytes were suspended in a physiological salt solution or a salt solution in which 80 mM
TEA-Cl replaced part of the NaCl. After 5 min at 37°C they were treated with leukocidin and
the response of the cell determined after 10 min.

Figure 6. Time course of the inhibition of calcium up-
take by TEA. Leukocytes in Hanks BSS or Hanks BSS in
which 80 mM TEA-Cl replaced the equivalent amount
of NaCl were treated with leukocidin (0.2 µg/10⁶ cells)
and ⁴Ca added. The radioactivity incorporated in the
cells was determined.

Fig. 7 shows that TEA inhibits the efflux of potassium from cells treated
with leukocidin but not from cells treated with streptolysin O. TEA did not
inhibit the efflux of potassium from leukocytes treated with vitamin A (500
μg/10^8 cells). Table VII shows that TEA is a more effective inhibitor of potassium efflux than some other quaternary ammonium ions. If normal leukocytes were incubated in the absence of calcium, the potassium content fell, presum-

**Figure 7.** Effect of TEA on the release of K⁺ from leukocytes treated with leukocidin or streptolysin O. The leukocytes in Hanks BSS or Hanks BSS in which 80 mm TEA-Cl replaced the equivalent amount of NaCl were treated with leukocidin or streptolysin O and the K⁺ content of the centrifuged cell pellets determined.

| Leukocidin concentration (μg/ml) | TEA-Cl | TMA-Cl | Choline-Cl |
|----------------------------------|--------|--------|-----------|
|                                  | 60 mm  | 80 mm  | 110 mm    | 60 mm | 80 mm | 110 mm | 60 mm | 80 mm |
| 0.2                              | 72     | 85     | 92        | 40    | 52    | 59     | 40    | 49    |
| 0.5                              | 40     | 58     | 75        | 18    | 25    | 32     | 25    | 30    |

Leukocytes suspended in Hanks BSS or Hanks BSS in which some of the NaCl was replaced by the quaternary ammonium chloride were treated with leukocidin and the potassium content of the centrifuged cell pellets determined. The efflux due to leukocidin is the difference in the K⁺ content of leukocidin-treated and control cells. TMA is tetramethylammonium.

ably through increased leakage. TEA did not inhibit the loss of potassium from cells maintained in calcium-free media. If leukocidin was added to leukocytes in the absence of calcium a further decrease in the potassium content was
induced and TEA inhibited this. It thus appears that TEA blocks the pathway of potassium efflux that is activated specifically by leukocidin.

**B. THE ACTION OF TEA ON THE p-NITROPHENYL PHOSPHATASE**

The failure of 80 mM TEA to affect the activity of membrane p-nitrophenyl phosphatase in the presence of substrate has been recorded above. Na\(^+\) and K\(^+\) at this concentration inhibit the p-nitrophenyl phosphatase and it was possible that a nonspecific effect of ionic strength could mask a stimulating action of TEA. In order to test this, three preparations of sodium iodide-treated membranes were maintained at room temperature for 20 min alone or with 80 mM TEA and then diluted 20-fold before assay. No change in the p-nitrophenyl phosphatase activity was found at pH 5, 6.2, or 7.5 with membranes tested alone or with 5 mM magnesium or 5 mM magnesium and 10 mM potassium. If membranes were treated with leukocidin in the presence of 80 mM TEA the stimulation by leukocidin was not prevented, providing further evidence that TEA does not act directly on leukocidin.

**DISCUSSION**

The cation-sensitive phosphatase and the ATPase of the leukocyte membrane are different from those of other cells. The leukocyte ATPase is insensitive to cations and to ouabain and the p-nitrophenyl phosphatase and its potassium-sensitive component both have a pH dependence different from the corresponding activities in the electric organ (13) or brain (17). It is not clear whether the leukocyte p-nitrophenyl phosphatase activity is due to a single enzyme. The appearance of peak activity at more than one pH value need not be taken as evidence for more than one enzyme. In the membrane the enzyme is in a solid state and its activity can be altered by conformational changes in neighboring molecules. The potassium-sensitive component of the p-nitrophenyl phosphatase is only active between pH 6.0 and pH 8 but treating the membranes with sodium iodide or leukocidin alters the activity at pH 5 as well and it is possible that the activity over the whole range 4.5–7.5 is due to a single enzyme.

The relationship between the cation-sensitive ATPase and the p-nitrophenyl phosphatase in membranes has not been clear. Albers, Rodriguez, and de Robertis (19) succeeded in partially separating the ATPase and acyl phosphatase of brain microsomes by differential centrifugation, and Albers and Koval (13) suggested that the enzymes were distinct in the electric organ of *Electrophorus*. The nature of the cation-sensitive phosphatases of the leukocyte membrane supports the conclusion that, in part at least, the p-nitrophenyl phosphatase is distinct from the cation-sensitive ATPase. However, p-nitrophenyl phosphate might be hydrolyzed by the ATPase in tissues that possess it and this could well account for the high potassium-sensitive p-nitrophenyl
phosphatase activity, at neutral pH, which is characteristic of the electric organ or brain.

The mechanism of electrolyte control in the leukocyte is different from that in other tissues. Elsbach and Schwartz (11), using cooled leukocytes, showed that the potassium concentration was restored on incubation but the sodium content was only partly reduced. They concluded that sodium and potassium movements were not linked in the leukocyte. It is possible that in the leukocyte the sodium pump is defective. There is good evidence that the ouabain- and cation-sensitive ATPase is part of an ion pump in the cell membrane (15) and, by analogy, the potassium-sensitive, ouabain-sensitive, p-nitrophenyl phosphatase might be part of an electrogenic potassium pump in the leukocyte.

Leukocidin stimulates the p-nitrophenyl phosphatase of the leukocyte membrane under the conditions required to produce the cytotoxic effects in the cell and the concomitant inactivation of leukocidin in solution. It is probable that the phosphatase is the site of action of leukocidin on the isolated membrane and that the potassium pump is the site of action of leukocidin in the cell. The action of DFP has supported this. All the cytotoxic effects of leukocidin tested (but not those of streptolysin O or vitamin A) are enhanced by DFP yet the mechanism of the cellular responses is not changed. Woodin and Wieneke (4) concluded from this that DFP and leukocidin have identical sites of action in the leukocyte. Independent evidence that this is the potassium pump was provided by showing that DFP inhibited the reaccumulation of potassium by depleted leukocytes.

The argument applied to determine the site of action of DFP on the leukocidin-treated cell can also be applied to that of TEA. In the leukocyte TEA inhibits all the effects of leukocidin tested, not by affecting the mechanism of the cellular responses but by inhibiting a reaction stemming directly from the action of leukocidin. With TEA we do not have independent evidence that it acts on the potassium pump. It is possible that TEA has no action on the normal leukocyte and it is only when leukocidin is present that receptors for TEA are available. Independent evidence for an action of TEA on the potassium pump may become available when the status of triphosphoinositide is clearer. Leukocidin and TEA can interact with triphosphoinositide in a highly specific fashion (12, 18) and triphosphoinositide is the only substance found to inactivate leukocidin in a way similar to the cell. However, it has not been possible as yet to show directly that triphosphoinositide is a component of the potassium pump.

Although DFP and TEA modify all the effects of leukocidin on the cell they do not affect all the interactions of leukocidin with the isolated membrane nor do they alter the p-nitrophenyl phosphatase activity. They appear to inhibit or enhance reactions in the potassium pump that stem directly and specifically from the activation by leukocidin of the ion pathway in the cell. Woodin and
Wieneke have shown that the action of DFP on the leukocidin-treated cell is due to its acting as a nonionic detergent and concluded that the enhancement of leukocidin by DFP is due to stabilization of the ion pathway. The inhibition of leukocidin action by TEA could arise from its blocking the ion pathway. Such an explanation would be consistent with the effects of TEA on nerve.

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