

## Mechanism of Haemolysis by *Vibrio vulnificus* Haemolysin

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The haemolytic action of *Vibrio vulnificus* haemolysin (VVH) was compared to that of streptolysin O (SLO). Both were cholesterol-binding haemolysins, but differed in the release of haemoglobin (Hb). In the first step of haemolysis, the haemolysins were temperature-independently bound to the cholesterol site on the target erythrocyte membrane. This was followed by the rapid release of  $K^+$ , which is an intra-erythrocyte marker. Hb was then released, in different ways. In the case of VVH, Hb was released slowly after a relatively long lag, whereas with SLO, Hb was released as rapidly as  $K^+$ . Haemolysis by VVH was inhibited by the addition of 30 mM-dextran 4 (mean  $M_r$  4000), which is considered to be an effective colloid-osmotic protectant. The results therefore indicated that haemolysis by VVH (like that by *Escherichia coli*  $\alpha$ -haemolysin and *Staphylococcus aureus*  $\alpha$ -toxin) was caused by a colloid-osmotic mechanism. Both  $K^+$  and Hb release caused by VVH proceeded temperature-dependently, and the membrane fluidity of liposomes prepared with lipids extracted from sheep red blood cell membranes increased above 20 °C. These results suggest that the temperature-dependence of the haemolysis by VVH is due to the requirement for an increase in the membrane fluidity during the formation of a transmembrane pore.

### INTRODUCTION

In the last decade, several new *Vibrio* species have been recognized as possible causes of human disease (Blake *et al.*, 1980; Tison & Kelly, 1984); *Vibrio vulnificus* (Roland, 1970) causes two types of infection: septicaemia and wound infections (Azuma *et al.*, 1984; Blake *et al.*, 1980). This *Vibrio* produces many substances associated with pathogenicity, one of which is a haemolysin (VVH) that can cause disruption of the membranes of most mammalian erythrocytes (Gray & Kreger, 1985). It possesses cytotoxic effects against Chinese hamster ovary cells, has the ability to accelerate vascular permeability in guinea-pig skin and shows a lethal action against mice (Kreger & Lockwood, 1981).

Haemolysis caused by bacterial cytotoxins may be of two types. Colloid-osmotic haemolysis is caused by *Staphylococcus aureus*  $\alpha$ -toxin (Füssle *et al.*, 1981), *Escherichia coli*  $\alpha$ -haemolysin (Bhakdi *et al.*, 1986), *Aeromonas hydrophila* aerolysin (Howard & Buckley, 1982) and others. Non-colloid-osmotic haemolysis is caused by SH-activated cytotoxins such as streptolysin O (SLO) (Bhakdi *et al.*, 1985; Niedermeyer, 1985), perfringolysin O (Mitsui & Hase, 1983) and others. In colloid-osmotic haemolysis, the initial pore size of the erythrocyte membrane formed by the haemolysins is small. The effective diameter of the pore size formed by *E. coli*  $\alpha$ -haemolysin is about 2–3 nm (Bhakdi *et al.*, 1986) and that of lesions present in the membrane after treatment with *S. aureus*  $\alpha$ -toxin is 2.5 nm (Füssle *et al.*, 1981). It is generally considered that molecules, such as positively-charged ions, cannot pass through the membrane. However, pores formed by the haemolysins can allow such molecules to permeate the cells. If haemoglobin (Hb), with an effective diameter of 4.8 nm, cannot pass through the pores formed by the

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; Hb, haemoglobin; HU, haemolytic unit; SLO, streptolysin O; VVH, *Vibrio vulnificus* haemolysin.

haemolysins, its release in haemolysis could have occurred by cell lysis. In a first step the haemolysin is envisaged to bind, either non-specifically to the target membrane (Cassidy & Harshman, 1976) or to specific receptors (Kato & Naiki, 1976), after which the haemolysin molecules form pores on the membrane through which ion leakage occurs leading to osmotic swelling and finally to the release of Hb when the cells are lysed (Füssle *et al.*, 1981). In non-colloid-osmotic haemolysis produced by SH-activated haemolysins, the initial pore size (30–40 nm) is large enough to allow the Hb molecules to pass through the membrane (Bhakdi *et al.*, 1985; Mitsui & Hase, 1983; Niedermeyer, 1985). Hb release follows the same pattern as the release of small molecules such as  $^{86}\text{Rb}^+$  (Duncan, 1974). The SH-activated haemolysins have common characteristics, being inactivated by cholesterol or mild oxidation and reactivated with SH-compounds, and possess immunological cross-reactivity.

As previously reported, the action of VVH was inhibited by the addition of cholesterol as in SH-activated cytolysins, e.g. SLO, perfringolysin O, etc.; however, VVH was not activated by thiol compounds, and did not have a common antigenicity with SLO (Miyoshi *et al.*, 1985; Shinoda *et al.*, 1985). It was therefore considered that the modes of action of VVH and SLO were different in spite of their common cholesterol-binding property. The present paper shows the difference in haemolytic action between VVH and SLO, and their different modes of action.

## METHODS

**Bacterial strain and cultivation.** A virulent strain of *V. vulnificus* (CDC B3547) producing haemolysin with high efficiency was used (Shinoda *et al.*, 1985); it was kindly supplied by the Osaka Prefectural Institute of Public Health. The strain was cultivated in heart infusion diffusate broth (Difco) for 7 h at 37 °C.

**Purification of VVH.** Crude haemolysin was obtained from the culture filtrate by ultrafiltration and ammonium sulphate fractionation as described by Gray & Kreger (1985). VVH was purified from the crude preparation by column chromatography on phenyl-Sepharose CL-4B (Pharmacia) by the method of Gray & Kreger (1985) and fast protein liquid chromatography (FPLC; Pharmacia). The FPLC was performed as follows. The partially purified VVH obtained from the hydrophobicity column chromatography was loaded on a Mono Q anion-exchange column (5 × 50 mm) equilibrated with 10 mM-Tris/HCl buffer (pH 7.5) (Miyoshi *et al.*, 1986). The column was washed with the 10 mM-Tris buffer followed by gradient elution with 0–0.5 M-NaCl solution in the 10 mM-Tris buffer. VVH was obtained as a single peak eluting at around 0.2 M-NaCl.

**SLO preparation.** Freeze-dried SLO (Nissui Pharmaceutical Co.) was used. The SLO solution (5 ml) was dialysed for 6 h against water (1 l) to remove  $\text{K}^+$ , then adjusted to the osmotic pressure of the erythrocyte suspension by the addition of NaCl (4.5 mg).

**Measurement of  $\text{K}^+$  released from erythrocytes.** A  $\text{K}^+$ -sensitive electrode, made with polyvinyl chloride-based membrane (Katsu *et al.*, 1986) was used.

**Assay of haemolytic action.** Haemolytic action was assayed by measuring the Hb released from sheep red blood cell (SRBC) suspensions treated with haemolysin (Shinoda *et al.*, 1985).

**Effect of osmotic protectants on haemolysis.** This was tested according to the method of Bhakdi *et al.* (1986). SRBC suspensions (1%, v/v) were prepared in a 12.5 mM-phosphate buffer (pH 7.2) containing 90 mM-NaCl and 45 mM-KCl, each containing one of the following osmotic protectants at 30 mM: sucrose, raffinose, inulin (Wako Pure Chemical Industries Co.) or dextran 4 (mean  $M_r$  4000; Serva). One-eightieth volume of haemolysin solution (800 HU  $\text{ml}^{-1}$ ) was added to the SRBC suspension (1 ml). Haemolysis was recorded for 30 min incubation at 37 °C. The following values were used for the mean molecular diameter of the osmotic protectants (Scherrer & Gerhardt, 1971): sucrose, 0.9 nm; raffinose, 1.1 nm; inulin, 2.8 nm; dextran 4, 3.5 nm. To test the effect of VVH concentration on pore formation, various amounts of VVH (2, 10 or 100 HU) were added to the above incubation system containing SRBC and osmotic protectants.

**Measurement of SRBC membrane fluidity.** Extraction of lipids from SRBC membranes was by the method of Rose & Oklander (1965). A portion (200 µg) of the extracted membrane lipids was solubilized in 2 ml chloroform/methanol (2:1, v/v), and 1,6-diphenyl-1,3,5-hexatriene (DPH) (0.5 µg) was added. Solvent was completely evaporated under reduced pressure. The lipid containing DPH was suspended in 10 mM-Tris buffer (1 ml), and the liposomes thus constructed were used to measure the membrane fluidity (Katsu *et al.*, 1986). VVH-induced changes in the phase transition temperature of the liposome suspension were measured by the fluorescence polarization technique (Hartmann *et al.*, 1978; Katsu *et al.*, 1984).

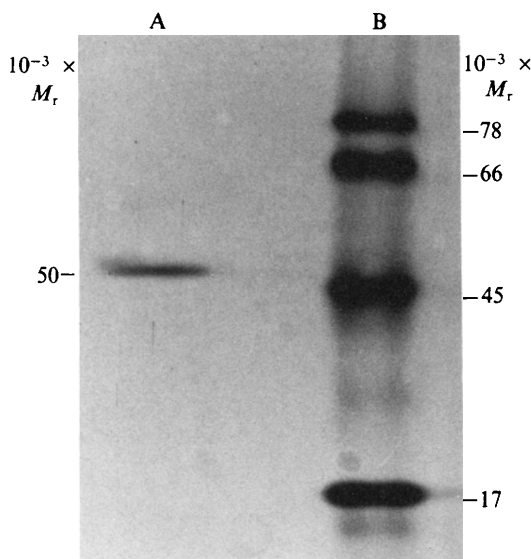


Fig. 1. SDS-PAGE of purified VVH. A, silver-stained gel loaded with purified VVH (0.4 µg); B,  $M_r$  markers – from top, ovotransferrin, bovine serum albumin, ovalbumin and myoglobin.

## RESULTS

### *Purification of VVH and measurement of $K^+$ and Hb released from SRBC treated with VVH or SLO*

The final preparation of VVH showed a single band in SDS-PAGE according to the modified method of Laemmli (1970) (Gray & Kreger, 1985). The  $M_r$  calculated from the SDS-PAGE was approximately 50 000 (Fig. 1).

SRBC (2.5%, v/v) suspensions in 10 mM-Tris buffer containing 140 mM-NaCl (pH 7.5) were each treated with VVH (10 HU ml<sup>-1</sup>) or SLO (10 HU ml<sup>-1</sup>). A rapid release of intracellular  $K^+$ , completed within 5–6 min, was observed with both haemolysins (Fig. 2a, b). The lag period of the  $K^+$  release was about 0.5 min. VVH treatment produced slow Hb release from SRBC and the release was completed after 30 min incubation. The Hb release from SLO-treated SRBC, like the  $K^+$  release, was completed within 5–6 min. The lag period of Hb release from VVH-treated SRBC was longer (about 1.5 min) than that observed with SLO. Furthermore,  $K^+$  and Hb release from erythrocytes treated with VVH only occurred at temperatures above 15 °C, indicating temperature-dependent release.

### *Measurement of the fluidity of the liposome membrane constructed with SRBC-membrane lipids*

The index of refraction of fluorescence of DPH incorporated in liposome membranes decreased with increase in temperature (Fig. 3); the slope changed at 20 °C, suggesting that the membrane fluidity increases above 20 °C. Addition of VVH did not affect the membrane fluidity.

### *Osmotic protection experiments*

SRBC (1%, v/v) suspensions containing various osmotic protectants were incubated with VVH (10 HU ml<sup>-1</sup>) or SLO (10 HU ml<sup>-1</sup>). Neither sucrose nor dextran inhibited SLO-dependent haemolysis. The effect of the protectants in VVH-dependent haemolysis varied with their molecular size. Sucrose and raffinose had no inhibitory effect on the VVH-dependent haemolysis, inulin had considerable inhibitory effect (80–90%), and dextran 4 completely inhibited the haemolysis (Fig. 4). When VVH-treated SRBC in which haemolysis was inhibited

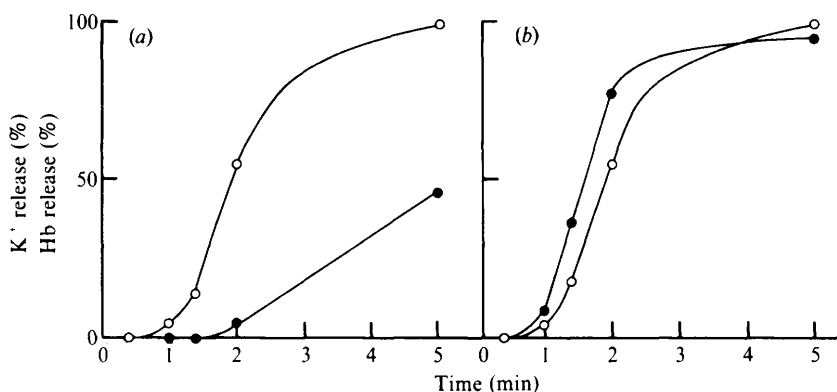


Fig. 2. Release of K<sup>+</sup> (○) and Hb (●) from sheep red blood cells treated with VVH (a) or SLO (b) (each 10 HU ml<sup>-1</sup>). Each haemolysin was incubated with SRBC suspension (2.5%, v/v) at 23 °C. A K<sup>+</sup>-sensitive electrode was used to determine K<sup>+</sup> released. Hb release was determined by measurement of absorbance at 540 nm.

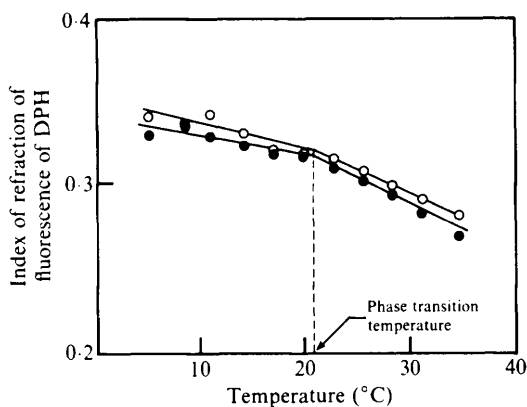


Fig. 3. Change in the degree of polarization of liposomes constructed with SRBC-membrane lipids before (●) and after (○) addition of VVH.

by dextran were collected by centrifugation, washed with dextran-containing buffer and resuspended in saline, complete haemolysis reappeared within 10–15 min on incubation at 37 °C. This suggested that VVH was able to bind to SRBC in the presence of dextran. Moreover, K<sup>+</sup> release from VVH-treated SRBC was observed even when the presence of dextran 4 inhibited haemolysis (Fig. 5), suggesting that the protectants did not affect pore formation in the erythrocyte membrane by VVH. These results also indicated that haemolysis by VVH was caused via a colloid-osmotic mechanism, and that the effect of the protectants in VVH-dependent haemolysis was not changed by VVH concentration.

#### DISCUSSION

Haemolysis by VVH followed a two-step process: a temperature-independent membrane-binding step and a temperature-dependent cell disruption step (Shinoda *et al.*, 1985; Gray & Kreger, 1985). In the latter step, both K<sup>+</sup> and Hb release were observed above 15 °C, but not below 10 °C. The membrane fluidity increased above 20 °C irrespective of addition of VVH (Fig. 3). The temperature dependence of the cell disruption step seems to be due to the

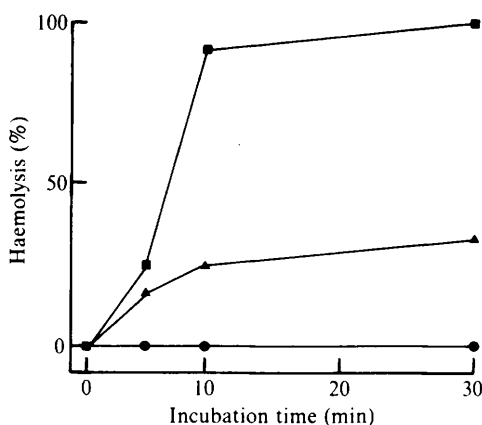


Fig. 4

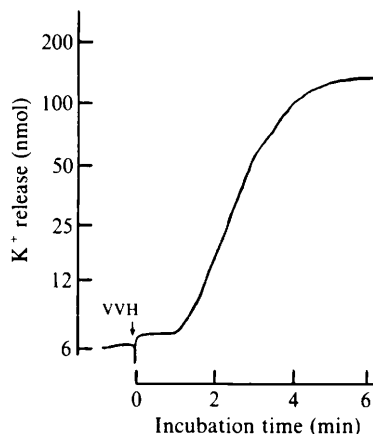


Fig. 5

Fig. 4. Effect of osmotic protectants on the haemolysis induced by VVH. SRBC (1%, v/v) suspensions containing 30 mM-sucrose (■), 30 mM-inulin (▲), or 30 mM-dextran 4 (●) were incubated with VVH (10 HU ml<sup>-1</sup>) at 37 °C.

Fig. 5. Release of K<sup>+</sup> from SRBC, suspended at 37 °C in saline containing 30 mM-dextran 4, after treatment with VVH (10 HU ml<sup>-1</sup>).

requirement for high temperature for increasing the membrane fluidity to allow transmembrane pore formation.

The difference in the time taken for K<sup>+</sup> and Hb release caused by VVH, i.e. a short lag period with rapid K<sup>+</sup> release and a relatively long lag period with slow Hb release, was in contrast to the results with SLO (Fig. 2). These results suggested that the temperature-dependent cell disruption step was further divided into two stages: a change in membrane permeability (transmembrane-pore formation) and an erythrocyte-bursting stage.

The intracellular K<sup>+</sup> could not pass through the normal SRBC membrane, but was able to permeate after the formation of pores due to VVH, although Hb could not be released through the pore at this stage (Fig. 5). Therefore, an electrolyte such as Na<sup>+</sup>, present in the extra-erythrocyte solution for maintaining the osmotic pressure, may enter the erythrocytes depending upon its concentration gradient. Consequently, the intracellular osmotic pressure of erythrocytes will be elevated, and water may enter the cells causing them to burst and release Hb (colloid-osmotic haemolysis).

Haemolysis by VVH was effectively inhibited by dextran 4; inulin showed a considerable inhibitory effect and sucrose and raffinose had no effect. It was therefore assumed that the effective diameter of the initial membrane pores formed by VVH was around 3 nm, which allowed the penetration of small molecules but not of large molecules such as Hb (effective diameter 4.8 nm). Moreover, it appeared that the size of the pores formed by VVH was not changed by VVH concentration, because the effect of the protectants in VVH-dependent haemolysis was not affected by VVH concentration. Inhibition of the haemolysis by osmotic protectants, e.g. dextran, suggested that haemolysis by VVH was produced in a colloid-osmotic manner similar to that of *S. aureus*  $\alpha$ -toxin or *E. coli*  $\alpha$ -haemolysin. Haemolysis by SLO was due to the formation of transmembrane channels of large pore size (Bhakdi *et al.*, 1985; Niedermeyer, 1985) and was not inhibited by the osmotic protectants.

When the erythrocyte membrane ghosts were observed by electron microscopy after complete haemolysis by VVH, 10–40 nm pores were observed (unpublished data). This suggests that large membrane pores were finally formed in the erythrocyte-bursting step.

Katoh (1979) reported that the change in membrane permeability caused by *S. aureus*  $\alpha$ -toxin was induced by a change in membrane-bound enzyme activity. The change in membrane

permeability by the haemolytic action of VVH may similarly involve such biological factors.

These experiments clearly show that haemolysis by VVH was produced in a colloid-osmotic mechanism, similar to the haemolysis caused by *E. coli*  $\alpha$ -haemolysin, *S. aureus*  $\alpha$ -toxin and others. VVH also possessed cholesterol-binding ability. In haemolysis caused by other cholesterol-binding haemolysins, such as SLO or perfringolysin O, a colloid-osmotic mechanism was not observed. It can therefore be concluded that VVH is a unique haemolysin with cholesterol-binding ability like SLO, but with a different mode of action in the release of Hb.

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