Action of Citrinin on Liposomes

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Citrinin, a mycotoxin, was studied for its effect on artificial membranes (liposomes). Of the three (net positive, negative and neutral) liposomal preparations tested, the membrane carrying a net positive charge was preferentially acted upon by citrinin and in this case even the lowest concentration was found to be sufficient for causing a high degree of damage. This is the first report of evidence that citrinin has a direct effect on membranes. It was shown to exhibit concentration dependency in its lytic activity on artificial membranes leading to leaching of trapped $^{45}Ca^{2+}$ ions.

Introduction

Citrinin is a benzopyran compound produced by *Penicillium citrinum*, whose occurrence in soil is known [1]. Its toxicity to animals has been studied [2] and its effect on seed germination and composition have been reported [3]. Its phytotoxic effects have also been demonstrated in cucumber, cotton, beans and sorghum [4, 5]. This phytotoxic compound causes an increased permeability in beet root slices indicating membranes as the possible site of action [6].

The chemical and functional complexity of membranes and the difficulty of isolating membranes while retaining their structural integrity have led to the development of model systems. It is used to understand the relationship between membrane structure and function and action of membrane-acting substances.

The influence of citrinin upon liposomes was therefore investigated and some of the observations are reported in this communication.

Materials and Methods

Pure citrinin crystals were isolated from static liquid cultures of *P. citrinum* grown for 25 days at room temperature [2]. The composition of the medium is already reported [7]. Precipitation of citrinin was achieved by acidification of the culture filtrate to pH 1.5, using 6 N HCl. The precipitate was then filtered, dried, taken up in absolute ethanol and crystallized. Recrystallization from the same solvent was done thrice. The physico-chemical properties were found to be identical to those of an authentic sample obtained from Prof. O. R. Rodig, Department of Chemistry, University of Virginia, USA. Solutions were prepared by dissolving the crystals in alkaline pH (8.5) and then adjusting to pH 7.0. Distilled water adjusted to pH 7.0 was used as control. Phosphatidyl choline was obtained as a crude preparation from Biochemicals Unit, V. P. Chest Institute, India and purified by chromatography over alumina and silica gel. $^{45}CaCl_2$ (19.5 mCi/mg Ca^{2+}) was obtained from Atomic Division, BARC, India.

Liposomes were prepared using phosphatidyl choline (PC), stearic acid and stearylamine in different molar ratios. 10, 15, 20 mol-% of either stearic acid or stearylamine was added to PC to give the negative or positive charge to the membrane. Lipids in chloroform solution were added to a small glass bottle and dried *in vacuo* to form an uniformly thin film. 1 mM $CaCl_2$ containing 2 μCi of ^{45}Ca in 0.01 M Tris-HCl buffer (pH 7.0) was added to yield a total lipid concentration of 10 μmol/ml. After the lipid film was removed by gentle shaking, the suspension was disrupted by sonic oscillation for 20 min. The milky suspension was carefully layered on top of a Sephadex G-50 column. The liposomes were then eluted from the

Abbreviation: PC, Phosphatidyl choline.

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column with 0.01 M Tris-HCl buffer (pH 7.0). Subsequently 1 ml samples of liposomes were incubated with various molar concentrations of citrinin ranging from 10^{-3} to 10^{-8} for 15 min at room temperature. Then the samples were dialysed against 10 ml of 0.01 M Tris-HCl buffer, pH 7.0. Leakage from liposomes through the dialysis sacs into surrounding fluid was determined at 30 min by counting $^{45}Ca^{2+}$ in a Liquid Scintillation Counter (ECIL, India). Leakage was expressed as percentage over control.

Results and Discussion

Amount of $^{45}Ca^{2+}$ trapped in various liposomes systems is presented in Table I.

The action of citrinin on the release of $^{45}Ca^{2+}$ in positively charged, neutral and negatively charged liposomes is shown in Figs 1 and 2. The results indicate that the leakage of the trapped marker from the liposomes carrying a net positive charge is high even when treated with the lowest concentration of toxin. The action of citrinin on negatively charged liposomes shows low amount of leakage and a saturation effect. Moreover, the leakage is enhanced, if the net positive charge of the membrane is increased whereas, the leakage decreases with increasing net negative charge.

PC liposomes do not carry net charge and are neutral. The action of citrinin on these liposomes is shown in Figs 1 and 2. The leakage of the trapped marker in the neutral liposomes is increased with increase in toxin concentration.

The results presented above document the capacity of citrinin to damage artificial phospholipid vesicles. The PC liposomes with stearylamine are more sensitive to citrinin than liposomes prepared with PC and stearic acid. The results indicate that a net positive charge may be essential for the higher interaction of citrinin with membranes.

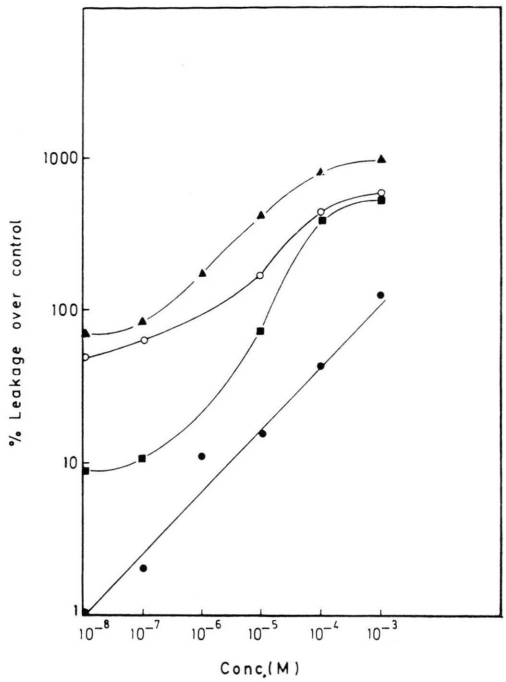

Fig. 1. $^{45}Ca^{2+}$ efflux from neutral (PC) and positively charged (PC: Stearylamine) liposomes. $^{45}Ca^{2+}$ loaded PC liposomes were prepared and the release of label with increasing citrinin concentration was determined. $^{45}Ca^{2+}$ loaded positively charged liposomes were prepared with different molar ratios of PC and Stearylamine. ●, PC; ■, 9:1 PC: Stearylamine; ○, 8.5:1.5 PC: Stearylamine; ▲, 8:2 PC: Stearylamine. Varying concentrations of citrinin were added and the amount of $^{45}Ca^{2+}$ release was determined.

Table I. Amount of Ca^{2+} trapped in various liposome systems.

Liposome preparation	$^{45}Ca^{2+}$ present in liposomes cpm/μmol lipid	% trapped
PC	13 451	6.2
PC : Stearylamine 9 : 1	9 240	4.2
PC : Stearylamine 8.5 : 1.5	11 132	5.0
PC : Stearylamine 8 : 2	13 464	6.1
PC : Stearic acid 9 : 1	25 300	11.5
PC : Stearic acid 8.5 : 1.5	33 000	15.0
PC : Stearic acid 8 : 2	45 540	20.7

Each dialysis bag contained 4 μmol of lipid. Liposomes were prepared and Ca^{2+} leakage was determined as explained in Materials and Methods.

This is confirmed by the fact that anionic citrinin has a very low influence on the negatively charged liposomes even at high concentrations. It is known that mutual repulsive forces between like charges of surfactant and target membranes might block the leakage [8]. It is interesting to note that polymyxin B has been shown to require negative charge for induction of membrane sensitivity [9] and on the contrary the action of *Prymnesium* toxin does not depend on the net charge on the membranes [10]. As detailed above, citrinin, apart from showing a concentration dependency exhibits a preference for a net positive charge on membranes to exert an effective damage.

This is the first report of evidence that citrinin may have a direct effect on membranes. This is

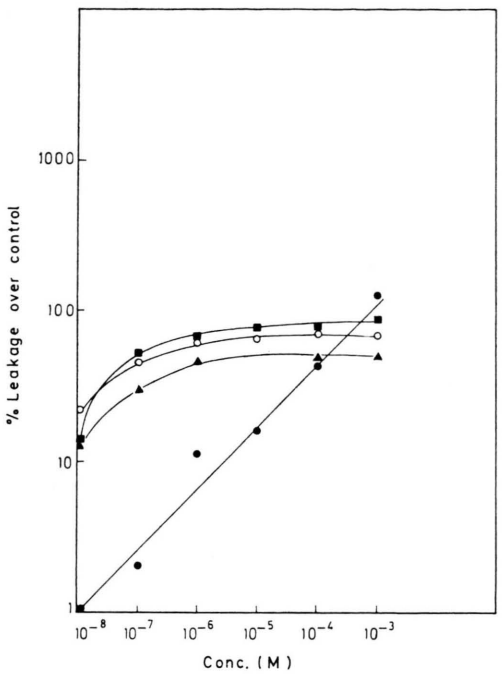

Fig. 2. $^{45}Ca^{2+}$ efflux from neutral (PC) and negatively charged (PC: Stearic acid) liposomes. $^{45}Ca^{2+}$ loaded PC liposomes were prepared and the release of label with increasing citrinin concentration was determined. $^{45}Ca^{2+}$ loaded negatively charged liposomes were prepared with different molar ratios of PC and Stearic acid. ●, PC; ■, 9:1 PC: Stearic acid; ○, 8.5:1.5 PC: Stearic acid; ▲, 8:2 PC: Stearic acid. Varying concentrations of citrinin were added and the amount of $^{45}Ca^{2+}$ release was determined.

further supported by our results obtained with sheep erythrocytes which exhibit a cent percent leakage of hemoglobin (2% SRBC) at all concentrations of citrinin employed.

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