Phytochemistry, Vol. 23, No. 3, pp. 543–547, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00
Pergamon Press Ltd.

DESTABILIZATION OF LIPOSOME MEMBRANES BY THE STEROIDAL GLYCOALKALOID α-TOMATINE

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(*Revised received* 15 *July* 1983)

Key Word Index—Liposomes; phospholipid; sterol; steroidal alkaloids; α-tomatine; membrane disruption.

Abstract—The effect of the steroidal glycoalkaloid α-tomatine on the leakage of peroxidase from liposomes was studied. At pH 7.2, the optimum pH for disruption, tomatine had little effect at concentrations less than 10 μM but was increasingly disruptive at concentrations of 10–100 μM. Liposome destabilization was pH-dependent declining with decreasing pH until at pH 5 lysis was achieved only at tomatine concentrations of 500–1000 μM. At all pH values tested (pH 5–8), tomatine caused significant disruption only if the membrane contained sterol. The extent of membrane damage was correlated with the concentration of sterol in the liposomes but not with the nature of the sterol or of the phospholipid. These findings are inconsistent with claims that surface glycosidases, which convert the glycoside to the aglycone, are prerequisites for tomatine action and that the aglycone is the active moiety.

INTRODUCTION

The steroidal glycoalkaloid α-tomatine, which is found in the tomato plant and other solanaceous species, is toxic to a wide range of organisms, a phenomenon both interesting and poorly understood [1]. Explanation of its pharmacological action on mammals [2] has been hindered by the complex nature of both the target organism and the physiological effects evoked, although it is widely considered that impairment of the membranes of strategic nerve and muscle cells is primarily responsible. Studies with simpler systems such as individual animal cells [3], fungal tissues [4] and plant organelles [5] have confirmed the ability of α-tomatine to alter membrane integrity and cause lysis.

The lytic action of steroidal alkaloids was earlier attributed to their amphipathic nature and detergent properties [6] but, following the report that tomatine is able to complex in a highly specific manner with 3β-hydroxy-sterols *in vitro* [7], it became apparent that binding of the glycoside to membrane sterols and destabilization of the lipid bilayer could often better explain the observed toxicity. Such a mode of action, which is similar to that of the polyene antibiotics [8], finds support in work from a number of areas, e.g. the similar pH dependence of sterol binding and toxicity [9], the reduced sensitivity of sterol-free (or virtually so) bacteria and species of *Pythium* and *Phytophthora* [4], the similar differential susceptibility of plant organelles to tomatine and polyenes [5], and the much reduced toxicity of hydrolysis products which do not bind sterols [9].

Hydrolysis of steroidal glycoalkaloids has thus been widely considered a detoxification mechanism, but more recently it was proposed [10–12] that the aglycone, and not the glycoside, is the principal active moiety, its release being brought about by the action of surface glycosidases. Since steroidal alkaloids appear to contribute to, or be important in, plant defences against pathogens and/or herbivores [13], how such compounds cause disruption of membranes obviously has important implications in plant pathology and ecology. We considered that the requirement for surface glycosidases might be usefully assessed and clarified using chemically defined synthetic membranes. This paper describes the effect of α-tomatine on the integrity of liposomes under a variety of experimental conditions.

RESULTS

The relationship between qualitative aspects of membrane composition and susceptibility to α-tomatine is shown in Table 1. Sterol-containing liposomes based on sphingomyelin sequestered approximately twice as much peroxidase as phosphatidylcholine (PC) liposomes and were much less leaky. In both types of liposome, enzyme sequestration was significantly enhanced by incorporation of sterol, but sterols only affected membrane permeability in sphingomyelin liposomes, supernatant enzyme being reduced from *ca* 48% to 6%. Different sterol treatments were, of necessity, carried out using different batches of liposomes prepared at different times and the resulting variability in response to tomatine rendered it difficult to draw conclusions regarding the relative importance of different membrane components. These experiments were carried out on three separate occasions but no consistent patterns emerged except that sterol was a prerequisite for tomatine action.

The role of sterols in the lysis of liposomes by tomatine was investigated using PC liposomes prepared with different quantities of cholesterol (Table 2). Sterol enhancement of enzyme sequestration and the resistance to lysis of sterol-free liposomes were again confirmed. Liposomes prepared using 1 mg sterol resembled sterol-free controls in both enzyme accumulation and susceptibility to tomatine. With 5 mg sterol, enzyme accumulation was similar to that of sterol-free controls but some disruption occurred. As the sterol concentration increased from 5 mg to 10

Table 1. Effect of tomatine on peroxidase leakage from liposomes containing different sterols and phospholipids

		Sterol											
		Cholesterol			Stigmasterol			Ergosterol			No sterol		
Treatment	Phospholipid	Total peroxidase act.*	% Act. in supernatant	% Act. in pellet	Total Peroxidase act.*	% Act. in supernatant	% Act. in pellet	Total peroxidase act.*	% Act. in supernatant	% Act. in pellet	Total peroxidase act.*	% Act. in supernatant	% Act in pellet
Control	PC	8.7	19.5	80.5	9.6	26.0	74.0	7.3	19.2	80.8	6.1	19.6	80.4
Tomatine	PC	9.2	52.0	48.0	10.5	51.4	48.6	7.8	69.2	30.8	5.2	15.4	84.6
Control	Sphingomyelin	17.1	6.3	93.6	27.4	10.6	89.4	16.3	7.4	92.6	3.1	48.4	51.6
Tomatine	Sphingomyelin	19.8	29.8	70.2	30.2	31.8	68.2	16.9	24.9	75.1	3.4	50.0	50.0

*Expressed as $\Delta A_{420}/\text{sec} \times 10^3$. Liposomes were treated with 150 μM alkaloid at pH 7.2 for 1 hr. Values are means of five replicates.

Table 2. Effect of cholesterol concentration on disruption of PC liposomes by tomatine

Cholesterol (mg)	Control			Tomatine		
	Total peroxidase act.*	% Act. in supernatant	% Act. in pellet	Total peroxidase act.*	% Act. in supernatant	% Act. in pellet
0	4.4	20.5	79.5	5.7	14.0	86.0
1	4.2	19.0	81.0	6.0	16.7	83.3
5	4.7	21.3	78.7	6.4	54.7	45.3
10	10.4	16.3	83.7	13.2	84.1	15.9
20	14.0	15.7	84.3	17.9	95.0	5.0

*Expressed as ΔA_{420}/sec $\times 10^3$. Sterol weight refers to the amount used in the original lipid mixture. Liposomes were incubated at pH 7.2 for 1 hr, with tomatine at 150 μM. Values are means of five replicates.

and 20 mg cholesterol the disruption due to tomatine intensified yielding supernatants with peroxidase activity, respectively, 250, 553 and 673% greater than that of the control supernatant.

The interaction between cholesterol concentration and tomatine concentration is shown in Fig. 1. Neither 50 μM nor 100 μM alkaloid had an effect on liposomes devoid of sterols. Treatment with 50 μM tomatine increased the proportion of enzyme lost from liposomes prepared with 5 and 10 mg cholesterol to 56 and 73%, respectively. Whereas with 100 μm tomatine there was no further effect on liposomes made with 5 mg cholesterol, leakage from liposomes made with 10 mg of the sterol increased to 87%. These results suggest membrane sterol concentration is an important, and potentially limiting, factor in the action of tomatine.

Disruption of living cells by tomatine is pH-dependent, with greater effects at higher pH values [9]. A similar pH effect occurs in the *in vitro* complexing of sterols by tomatine (Fig. 2) and in the disruption of liposomes by alkaloids (Fig. 3). Above pH 6 the permeability of liposomes increased but control values for total peroxidase accumulation were quite constant (5.3–5.8 units) over the pH range tested indicating that the tomatine data represent a pH-dependent effect of the alkaloid rather than an effect of pH on enzyme activity. Maximal effect of tomatine occurred around pH 7 at which approximately 86% of the enzyme was released into the supernatant. There was no further increase, and possibly a slightly reduced effect, at pH 8 but this was not due to any limitation by the sterol.

Cholesterol-based liposomes could be lysed by tom-

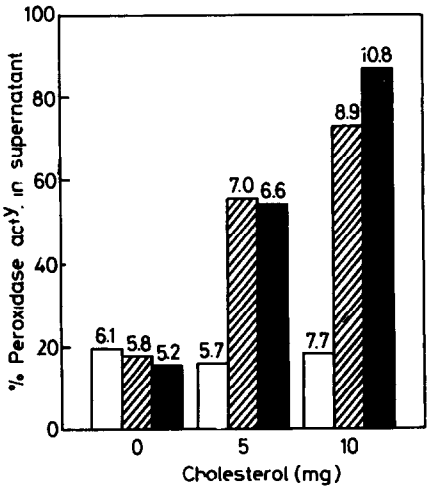

Fig. 1. Interaction between cholesterol level and tomatine concentration in peroxidase leakage from PC liposomes. Sterol weight refers to the amount used in the original lipid mixture. Open, hatched and filled columns represent zero, 50 μM and 100 μM tomatine, respectively. The figure at the top of each column is the total enzyme activity expressed as ΔA_{420}/sec $\times 10^3$. Reactions were carried out at pH 7.2 for 1 hr. Data are means of five replicates.

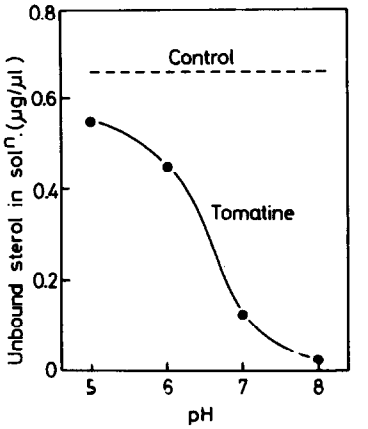

Fig. 2. Effect of pH on binding of tomatine to cholesterol *in vitro*. Solutions of 0.5 mM tomatine and 10 mM cholesterol in 96% ethanol were adjusted to pH with acid–base, mixed in the proportion of 4 ml : 0.2 ml, respectively and left at room temperature for 24 hr. Tubes were centrifuged at 3000 *g* for 15 min and 1 ml of supernatant removed and evaporated to dryness in an air stream at 50°. The residue was dissolved in 0.25 ml of a 0.04% solution of 5α-cholestane in ethyl acetate and the free (unbound) cholesterol concentration in a 1 μl aliquot determined by GLC, as previously described [26]. Control treatments lacked tomatine. Values are means of three replicates.

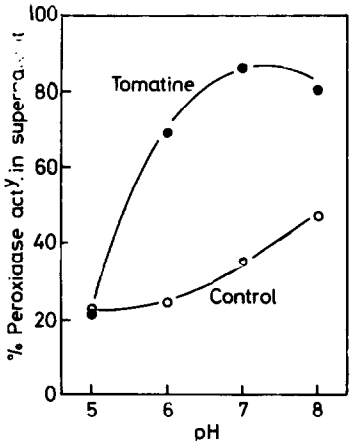

Fig. 3. Effect of pH on disruption of liposomes by tomatine. Liposomes were prepared with PC and cholesterol. Reaction mixtures were as in the Experimental except that Tris buffer (pH 7.2) was substituted by phosphate/citrate buffer at the appropriate pH. Treatment was for 1 hr and the tomatine concentration was 150 μM. Values are means of five replicates.

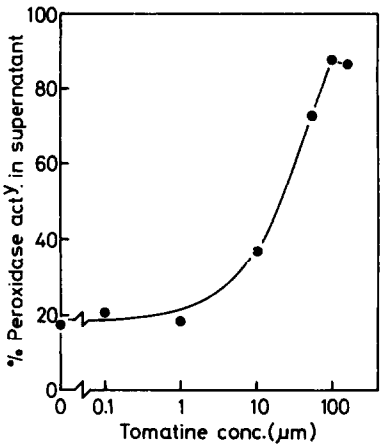

Fig. 4. Semi-log plot showing the effect of tomatine concentration on disruption of liposomes. Liposomes were prepared with PC and cholesterol and incubated at pH 7.2 for 1 hr. Values are based on five replicate determinations.

Table 3. Interaction of tomatine and sterol on peroxidase leakage from PC liposomes at low pH

Sterol	Tomatine concn (μM)	Total peroxidase activity*	% Activity in supernatant	% Activity in pellet
Cholesterol	0	6.4	21.9	78.1
Cholesterol	500	6.8	33.8	66.2
Cholesterol	1000	7.0	75.7	24.3
None	0	6.4	29.7	70.3
None	500	7.4	33.8†	66.2
None	1000	7.0	38.6‡	61.4

*Expressed as ΔA_{420}/sec $\times 10^3$.

†Significantly different from control at 5% level; ‡at 1% level.

Treatments were incubated in phosphate/citrate buffer (pH 5) for 1 hr. Values are means of five replicates.

atine at pH 5 when exposed to higher alkaloid concentrations (Table 3). At 500 μM, supernatant peroxidase activity increased by 64% whereas with 1000 μM the increase was 279%. With sterol-free liposomes there was a significant but much reduced effect of tomatine, with increases of only 32 and 42% at the two alkaloid concentrations.

The efficacy of tomatine against liposomes was studied at pH 7.2 using concentrations ranging from 0.1 to 150 μM (Fig. 4). A concentration of 1.0 μM or less proved ineffective, but at 10 μM enzyme leakage had increased by 98% and at 100 μM by 381% over control values. Increasing the concentration of tomatine to 150 μM did not bring about any further lysis and the low solubility of tomatine at pH 7.2 prevented the testing of greater concentrations.

DISCUSSION

Lysis of liposomes by tomatine exhibits features similar to those observed in natural membrane studies. The concentration range for activity at higher pH values (10–100 μM) lies within that reported to disrupt fungal hyphae [4], plant cells and organelles [5] and erythrocytes [3], the requirement for membrane sterols concurs with reports relating this factor to the susceptibility of fungal cells to tomatine [14], the pH dependence of susceptibility to tomatine is consistent with the response shown by erythrocytes [3] and fungal spores [9].

This evidence supports the hypothesis that tomatine disrupts membranes principally by interacting with their sterol component, which is known to be important in stabilizing membranes [15], thus causing a change in the permeability characteristics of the membrane. Sterols play a similar role in relation to the damage caused to natural and synthetic membranes by polyene antibiotics [16–20]. The fact that tomatine action is influenced by the sterol levels in membranes could explain the differential susceptibility to tomatine of, for example, erythrocytes, fungal cells and organelles such as mitochondria which have widely differing sterol contents.

Our data refute claims that glycosidase enzymes are a prerequisite for tomatine action and that the aglycone, tomatidine, is the moiety active in damaging membranes [10–12]. Obviously caution is necessary in extrapolating from liposomes to living cells, but the similar behaviour of these two systems in terms of the efficacy of the glycoside and the aglycone, pH effects and sterol requirement suggests that the comparison is valid. Surface glycosidases have been shown to hydrolyse tomatine in a number of fungi [21–24] but in all cases degradation was considered a detoxification, rather than an activation, mechanism.

Contrary to the findings of Demel *et al.* [20] with lipid monolayers and of Kleinschmidt *et al.* [25] with liposomes, no evidence was obtained (Table 1) that sterol type has a major influence on the susceptibility of liposomes to membrane-disrupting compounds. Our results are in agreement with studies on the effect of filipin on *Pythium irregulare* [16] but contrast with earlier *in vitro* work [26] which indicated that, of the sterols included here (Table 1), cholesterol was the least readily bound by tomatine. Kleinschmidt *et al.* [25] also reported a lack of correlation

between results obtained from *in vitro* experiments with stigmasterol and those obtained using liposomes containing that sterol. Thus extrapolations from liposomes to cells may be more valid than from an *in vitro* to a liposome system.

The need for a higher tomatine concentration to induce lysis at lower pH is consistent with findings with animal and plant cells [3, 5]. However, this paper provides the first indication that the action of tomatine at pH values around 5.0 is still largely sterol-dependent. The phenomenon may be explained by the lower affinity of tomatine for sterol at this pH (Fig. 2) being offset by increased concentration of the alkaloid. Interaction with sterols thus appears to be the principal mode by which tomatine disrupts liposome membranes over the pH range examined. Further studies are in progress to determine if the same mechanism is involved in the interaction between glycoalkaloids and cells.

EXPERIMENTAL

Preparation of liposomes. Cationic liposomes were prepared by a modification of the method of ref. [27] using 50 mg phospholipid (egg yolk PC—Sigma type VII-E or bovine brain sphingomyelin—Sigma), 10 mg sterol (cholesterol, stigmasterol or ergosterol—Sigma) and 2 mg stearylamine. These constituents were dissolved in 20 ml $CHCl_3$–MeOH (9:1) and the soln evapd to dryness under vacuum at room temp. The flask was flushed with N_2 and a 1.5 ml aliquot of soln (40 mg/ml) of horse-radish peroxidase (Sigma type II) in 0.2 M phosphate buffer (pH 7.2) added together with six 4 mm diameter glass beads. The lipid material was removed from the flask wall by agitating the beads with a vortex mixer and the resulting suspension was sonicated at 26 kHz for six 20 sec periods alternating with 20 sec pauses. The suspension was diluted to 5 ml with 0.05 M Tris–HCl buffer (pH 7.2) and centrifuged at 50 000 *g* for 30 min at 4°. The supernatant was discarded and the pellet resuspended in 5 ml Tris buffer and centrifuged twice more, as previously. The final liposome pellet was suspended in 1 ml Tris buffer (pH 7.2) and stored under N_2 at 4° until required. Liposomes were normally used immediately following preparation.

Microscopic examination. The appearance of the liposomes was studied using light- and electron microscopy. Nomarski interference contrast microscopy revealed numerous spherical bodies up to *ca* 6 μm diameter and often with a concentric appearance. For electron microscopy, liposomes were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 hr then post-fixed in 2% aq. OsO_4 for 1 hr. Sections were stained with either 1% aq. uranyl acetate or 1% aq. tannic acid, post-stained with lead citrate and examined in a Jeol 100S transmission electron microscope. Spherical, membranous bodies of varying sizes were apparent, usually 1–6 μm diameter and showing varying degrees of concentric lamellation.

Treatment with alkaloids. The reaction mixture consisted of 0.05 ml liposome suspension, 3.65 ml Tris buffer (pH 7.2) and 0.3 ml alkaloid (or control) soln. Tomatine (Sigma) was prepared as a 2 mM soln by dissolving 40 mg in 0.4 ml 0.1 M HCl and making to 20 ml with distilled H_2O. The pH of the soln was 3.8. The control soln was identical but lacked alkaloid. The final concn of tomatine in the reaction mixture was 150 μM. Reaction mixtures were incubated at 25° for 1 hr, followed by centrifugation at 50 000 *g* for 30 min at 4°. The supernatant (4 ml) was decanted and the pellet re-suspended in 4 ml Tris buffer (pH 7.2).

Assay method. The extent of liposome disruption was assessed by determining the proportion of peroxidase activity in the pellet and supernatant. For supernatants, a 0.025 ml aliquot was transferred to a 10 mm spectrophotometer cuvette followed by 2.5 ml 0.025 M soln of pyrogallol in 0.01 M phosphate buffer (pH 7.0), 0.3 ml distilled H_2O and 0.25 ml 1% H_2O_2. Assay of liposome (pellet) peroxidase was as for supernatants except that H_2O was substituted by 0.025% Triton X-100 to disrupt the liposome membrane. Both pyrogallol and H_2O_2 solns were used freshly prepared and the former was protected from light. Absorbance of the mixture at 420 nm was continuously recorded for *ca* 40 sec using a chart recorder operating at 20 sec/cm. The change in absorbance per sec (ΔA_{420}/sec) was calculated and used as an index of enzyme activity.

Replication and statistical analyses. Except where otherwise stated, all expts were carried out using five replicates and each expt conducted at least twice. Where necessary, statistical significance between means was determined using a *t*-test.

Acknowledgements—We wish to thank Mrs. A. Rijnenberg, Dr. H. Stebbings and Mr. G. Wakley, University of Exeter, for technical assistance, Nomarski microscopy and electron microscopy, respectively.

REFERENCES

1. Roddick, J. G. (1974) *Phytochemistry* **13**, 9.
2. Wilson, R. H., Poley, F. W. and DeEds, F. (1961) *Toxicol. Appl. Pharmacol.* **3**, 39.
3. Schlösser, E. (1975) *Z. Pflkr. Pfls* **82**, 476.
4. Arneson, P. A. and Durbin, R. D. (1968) *Phytopathology* **58**, 536.
5. Roddick, J. G. (1978) *J. Exp. Botany* **29**, 1371.
6. McKee, R. K. (1959) *J. Gen. Microbiol.* **20**, 686.
7. Schulz, G. and Sander, H. (1957) *Z. Physiol. Chem.* **308**, 122.
8. Norman, A. W., Spielvogel, A. M. and Wong, R. G. (1976) in *Advances in Lipid Research* (Paoletti, R. and Kritchevsky, D., eds.), Vol. 14, p. 127. Academic Press, New York.
9. Arneson, P. A. and Durbin, R. D. (1968) *Plant Physiol.* **43**, 683.
10. Segal, R., Shatkovsky, P. and Milo-Goldzweig, I. (1974) *Biochem. Pharmacol.* **23**, 973.
11. Segal, R. and Milo-Goldzweig, I. (1975) *Biochem. Pharmacol.* **24**, 77.
12. Segal, R. and Schlösser, E. (1975) *Arch. Microbiol.* **104**, 147.
13. Roddick, J. G. in *The Biology and Systematics of the Solanaceae* (D'Arcy, W. G. and Hawkes, J. G., eds.), in press.
14. Défago, G. (1977) *Ber. Schweiz Bot. Ges.* **87**, 79.
15. Demel, R. A. and de Kruyff, B. (1976) *Biochim. Biophys. Acta* **457**, 109.
16. Schlösser, E. and Gottlieb, D. (1966) *Z. Naturforsch.* **21**, 74.
17. Weber, M. M. and Kinsky, S. C. (1965) *J. Bacteriol.* **89**, 306.
18. Andreolli, T. E., Dennis, V. W. and Weigl, A. M. (1969) *J. Gen. Physiol.* **53**, 133.
19. Kinsky, S. C., Haxby, J., Kinsky, C. B., Demel, R. A. and van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta* **152**, 174.
20. Demel, R. A., van Deenen, L. L. M. and Kinsky, S. C. (1965) *J. Biol. Chem.* **240**, 2749.
21. Arneson, P. A. and Durbin, R. D. (1967) *Phytopathology* **57**, 1358.
22. Schlösser, E. (1975b) *Acta Phytopathol. Acad. Sci. Hung.* **10**, 77.
23. Verhoeff, K. and Liem, J. I. (1975) *Phytopathol. Z.* **82**, 333.
24. Ford, J. E., McCance, D. J. and Drysdale, R. B. (1977) *Phytochemistry* **16**, 545.
25. Kleinschmidt, M. G., Chough, K. S. and Mudd, J. B. (1972) *Plant Physiol.* **49**, 852.
26. Roddick, J. G. (1979) *Phytochemistry* **18**, 1467.
27. Magee, W. E., Goff, C. W., Schoknecht, J., Smith, M. D. and Clevian, K. (1974) *J. Cell Biol.* **63**, 492.