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COMPLEX FORMATION BETWEEN SOLANACEOUS STEROIDAL GLYCOALKALOIDS AND FREE STEROLS *IN VITRO*

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Key Word Index—*Lycopersicon* sp.; *Solanum tuberosum*; Solanaceae; complex formation; steroidal glycoalkaloids; α-tomatine; α-solanine; α-chaconine; sterols; cholesterol; sitosterol; stigmasterol; campesterol; ergosterol.

Abstract—Both the steroidal glycoalkaloid mixture from potato (α-solanine and α-chaconine) and pure α-tomatine are able to complex with the sterols cholesterol, sitosterol, stigmasterol, campesterol and ergosterol *in vitro*. The sterol-complexing ability of tomatine was greater than that of the potato alkaloids and more akin to that of the steroidal saponin, digitonin. With all three compounds, cholesterol was the least-readily bound sterol while binding to other sterols was of a similar order. Complex formation with tomatine was not markedly influenced by temperature, and with the aglycone tomatidine did not appear to occur at all.

INTRODUCTION

The membrane-lytic action and toxicity of steroidal glycoalkaloids such as α-tomatine, α-solanine and α-chaconine have been well documented [1–7] but the biochemical basis of their effects has not been satisfactorily explained. Recently, it has been suggested that the aglycone, liberated by the action of membrane-bound β-glycosidases, is the active form [8, 9], but this is still far from certain. There is some evidence that toxicity may be due to surfactant effects on cell membranes [1, 2], but available information points to membrane destabilization resulting primarily from complex formation between the glycoside and membrane sterols [10]. Schulz and Sander [11] were the first to show that tomatine forms an insoluble 1:1 molecular complex with 3β-hydroxy steroids such as cholesterol in ethanolic solution, and other steroidal alkaloids including α-solanine and α-chaconine also possess this ability [12]. Tomatine has been used for the preparation and estimation of cholesterol [13] and its ability to complex with, and remove from solution, cholesterol-[^{14}C] is the basis of an assay devised by Heftmann and Schwimmer [14].

The cholesterol-binding properties of tomatine and potato glycoalkaloids (PGA) could help explain their lytic action on cholesterol-rich erythrocyte membranes but it should be borne in mind that these alkaloids also cause leakage from and/or impairment of fungal hyphae [15], higher plant cells [16, 17] and plant cell organelles [18] which contain little or no cholesterol. These structures do contain reasonable amounts of other sterols, e.g. ergosterol in hyphae and sitosterol, stigmasterol and campesterol in plant cells, although the ability of these compounds to complex with steroidal alkaloids is not known. This work provides some information on this subject in relation to α-tomatine and PGA and, for comparison, to the steroidal saponin, digitonin.

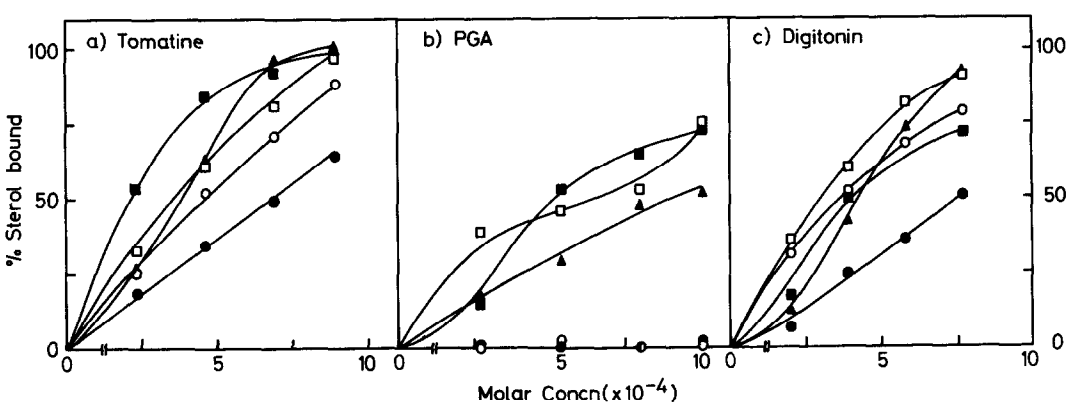

Fig. 1. Effect of steroid concentration on the proportion of sterol bound. Complex formation in 96% EtOH was assessed after at least 16 hr incubation by measuring free sterol remaining in solution. Cholesterol and sitosterol were [4-^{14}C]-labelled and were assayed by scintillation spectrometry; all other sterols were unlabelled and were assayed by GLC. Each point represents the mean of three replicate determinations. ●, Cholesterol; ○, sitosterol; □, campesterol; ■, stigmasterol; ▲ ergosterol.

Table 1. Complex formation between sterols, PGA and tomatidine

Sterol	Treatment	Supernatant (cpm/ml)	Sterol (mg) remaining in soln (by radioassay)	Sterol (mg) remaining in soln (by GLC)	% Sterol bound (GLC data)
Cholesterol-[4-^{14}C]	Control	743	0.68	0.57	0
	+ PGA	819	0.75	0.40	29.8
Sitosterol-[4-^{14}C]	Control	764	0.70	0.69	0
	+ PGA	874	0.80	0.34	50.8
Sitosterol-[4-^{14}C]	Control	884	0.81	0.87	0
	+ tomatidine	874	0.80	0.90	N.S.

Complex formation in 96% EtOH was assessed after at least 16 hr incubation. In each case alkaloid concn was *ca* 5×10^{-4} M. Each figure is the mean of three replicates.
N.S. = Not significant.

RESULTS

Figure 1 shows the proportion of sterol which was bound by varying amounts of the different steroids. With the exception of PGA with cholesterol and sitosterol, all other combinations of steroid and sterol showed evidence of complex formation. The binding of tomatine and digitonin to the various sterols was very much of the same order. In each case, sitosterol, campesterol and ergosterol were bound to a similar degree but cholesterol to a much lower degree (Figs. 1a and 1c). Digitonin binding to stigmasterol was much as for sitosterol, campesterol and ergosterol (Fig. 1c) but tomatine appeared to show slightly greater binding to this sterol (Fig. 1a). In contrast, of the sterols which did apparently complex with PGA, all appeared to do so much less readily than with tomatine or digitonin and there was some indication of reduced binding of PGA to ergosterol compared with stigmasterol and campesterol (Fig. 1b). On repeating the experiments using PGA (at one concentration only) with cholesterol and sitosterol and assaying sterol by both scintillation counting and GLC, it was found that, whereas the former method gave similar results to those previously obtained (with no evidence of binding), the latter method revealed a decline in the amount remaining of both sterols, indicating that some complex formation had taken place (Table 1). These apparently conflicting results probably arose from the greater solubility of PGA/sterol complexes, as shown by Tschesche and Wulff [12]. Because of this, complexes were not removed by centrifugation and thus contributed to the radioactivity of the sample, whereas GLC, being more specific, enabled changes in the concentration of free (cf. total) sterol to be detected. Consistent with the findings obtained with unlabelled sterols, PGA showed reduced complex formation with cholesterol and sitosterol compared with tomatine and digitonin but, as with tomatine and digitonin, cholesterol was the least readily bound of all the sterols. These binding patterns become more apparent when results are expressed as the concentration of steroid required to complex with 50% of the available sterol (Table 2). Based on the steroid:sterol ratio of 1:1 reported by Schulz and Sander [11], the consistently higher values obtained with cholesterol tend to suggest a lower affinity for this sterol. However, determination of actual binding constants would probably be necessary to ascertain if the steroids showed differential affinity for sterols other than cholesterol.

Table 2. Approximate concentrations of steroids required to complex with 50% of available sterol

Sterol	Steroid: Tomatine	PGA	Digitonin
Cholesterol	6.8	9.2*	7.2
Sitosterol	4.5	5.3*	3.6
Campesterol	3.5	6.5	2.9
Stigmasterol	2.1	5.3	4.1
Ergosterol	3.8	9.0	4.3

Values ($\times 10^{-4}$ M) were obtained from Fig. 1 except asterisked values which were derived by extrapolation of data in Table 1.

Complex formation between tomatidine (the aglycone of tomatine) and sitosterol-[^{14}C] was also studied and the results are shown in Table 1. No precipitate was observed and supernatants showed no reduction in radioactivity in tomatidine-treated tubes. GLC of the same supernatants confirmed that the concentration of free sitosterol was not affected by the presence of the aglycone.

The radioligand method of Heftmann and Schwimmer [14] for estimating tomatine involves briefly heating the mixture at high temperature for a few minutes (5 min at 90°) and cooling, firstly at room temperature for 1 hr, then overnight in a refrigerator. Although these treatments supposedly aid formation and separation of the complex *in vitro*, they do not help explain the *in vivo* action of tomatine in an aqueous environment and at ambient temperature. The need for temperature treatment for binding of tomatine to sitosterol-[4-^{14}C] was investigated firstly by treating mixtures at 10, 25, 37, 60 and 90° for 5 min then cooling as before. Results based on three replicates of each treatment showed no significant differences whatsoever, the mean residual sterol ranging from 0.72 to 0.76 mg in control tubes and from 0.33 to 0.40 mg in tomatine treatments (5×10^{-4} M). In a second series of experiments in which tubes were first treated at 25° for 5 min, it was found that subsequent overnight incubation temperature (4 or 25°) did not influence complex formation. Control values at both temperatures were in the range 0.51–0.53* mg residual sterol while tomatine-treated tubes (5×10^{-4} M) gave identical means of 0.14 mg* sterol.

Earlier attempts to investigate the time-course of complex formation were hindered by the temperature treatment steps in the procedure. Having shown that such treatment was not a critical factor, time-course

* By error slightly less than the usual 0.1 ml of sterol solution was added, hence the lower values.

experiments were conducted at room temperature and complex formation assessed by nephelometry. Only tomatine was used as PGA/sterol complexes were found to be mainly soluble even at alcohol concentrations as low as 70%. The experiment was conducted with the sterols used previously and the results are shown in Fig. 2. In all cases except for cholesterol, complex formation was detectable within the first minute. The results essentially confirm those reported in Fig. 1a with the cholesterol complex being the slowest to appear and all other complexes showing a similar pattern of formation. This correspondence in results, despite the use of different methods, tends to minimize the possibility that the nephelometry data reflected differences in solubility rather than binding. The possibility raised in Experiment 1 that stigmasterol might complex with tomatine more readily than the other sterols was not confirmed, suggesting that the difference shown in Fig. 1a was not significant. The levelling off of the ergosterol curve at a lower scale value was probably due to decline in the sterol concentration caused by light degradation [19]. When the experiment was repeated under more stringently-controlled light conditions, the curve for ergosterol levelled out at a similar scale value to the other comparable sterols.

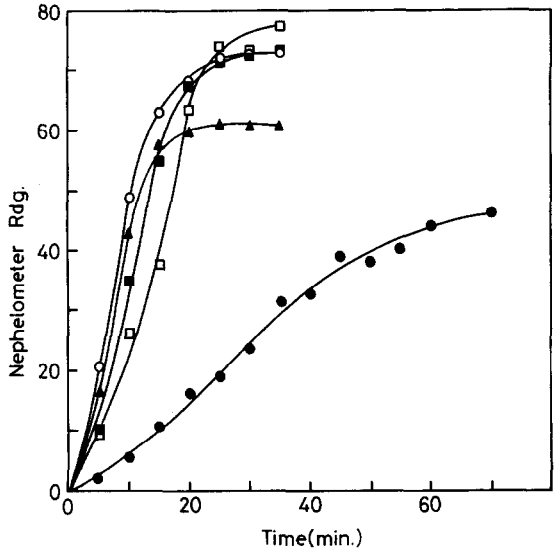

Fig. 2. Time-course of complex formation between tomatine and sterols. Tomatine and sterols in 96% EtOH were mixed and the formation of insoluble complex followed over a period of time by nephelometry. Controls lacked tomatine and showed zero readings throughout the experiment. Each point represents the mean of three replicates. ●, Cholesterol; ○, sitosterol; □, campesterol; ■, stigmasterol; ▲, ergosterol.

DISCUSSION

Evidence is presented that the steroidal alkaloids of tomato and potato are able to complex with the major plant and fungal sterols *in vitro*, findings which are consistent with those of Schulz and Sander [11] who showed that tomatine complexes with steroids possessing a 3β-hydroxyl group. The results reported here not only confirm the finding of Kabara *et al.* [13] that tomatine and digitonin bind cholesterol to a similar degree, but also extend it to other major sterols. They also concur with the observation by Tschesche and Wulff [12] that solanine and chaconine bind cholesterol less readily than tomatine or digitonin. However, at this stage, it is not certain whether reduced binding with PGA is due to a lower affinity for sterols or to a higher steroid: sterol binding ratio, or to both.

The structures of tomatine and PGA differ in a number of ways, e.g. tomatine is a tetraoside and PGA triosides; tomatidine has a saturated ring system while solanidine possesses a Δ^5 double bond; tomatidine is a secondary amine with N present in the F ring whereas solanidine is a tertiary amine with the N incorporated into a fused indolizidine moiety. These differences make it difficult to identify the molecular features which determine the degree of sterol binding and the solubility of the complexes. Nevertheless, the apparently greater sterol-complexing ability of tomatine than PGA is of interest in view of reports that tomatine is more toxic than solanine towards fungi and erythrocytes [1, 12, 20].

With the exception of ergosterol, which has an additional Δ^7 double bond, all the sterols used in this work share the same steroid nucleus with a 3β-hydroxyl group, C-18 and C-19 methyl groups, and a Δ^5 double bond. Thus, differential complexing with steroidal alkaloids can be seen to be a consequence principally of differences in the sterol side-chain structure. The only consistent side-chain difference between cholesterol and the other four sterols is substitution in the latter at C-24, campesterol and ergosterol possessing a C-24 methyl and stigmasterol and sitosterol a C-24 ethyl. (Stigmasterol and ergosterol also possess a Δ^{22} bond but results do not indicate any consistent contribution of this feature to binding.) It may be therefore that substitution at C-24 and/or the bulkiness of the sterol side chain enhances binding by steroidal alkaloids, although no conclusions can be drawn yet regarding the effect (if any) of the type of substituent at C-24. The size of the side chain of certain sterols has been identified as one of the structural features responsible for their membrane-stabilizing properties, and cholesterol, which has the least bulky side chain of the sterols tested, was found to be the most effective membrane stabilizer [21]. However, it seems unlikely that any direct or causal relationship exists between the greater effectiveness of cholesterol as a membrane stabilizer and its reduced binding to membrane destabilizers such as steroidal alkaloids.

The inability of tomatidine to complex with sitosterol is in keeping with a similar finding made by Arneson and Durbin [22] using cholesterol. Thus, although a certain toxicity has been ascribed to the aglycone [8, 9, 23], it seems unlikely that this is a consequence of, or related to, binding to membrane sterols.

That the steroids tested here are capable of complexing with plant sterols is at least compatible with their reported ability to disrupt plant cells and organelles [16–18, 24–26]. Nevertheless, a certain amount of caution must be exercised when considering possible correlations between alkaloid toxicity and sterol-binding capacity. In this respect, the reports of Schlösser [20], who found no strong correlation between haemolytic potential of various saponins and their affinity for cholesterol, and Kleinschmidt *et al.* [27], who showed that the polyene antibiotic filipin, although capable of binding to stigmasterol, did not disrupt stigmasterol-

containing liposomal membranes, should be borne in mind.

This work demonstrates complex formation between certain steroidal alkaloids and various free sterols *in vitro* but does not allow any conclusions to be drawn regarding sterol binding and its relative importance *in vivo*. This is an area still in need of clarification.

EXPERIMENTAL

Authentic α-tomatine was obtained from Koch-Light Laboratories, Colnbrook, Bucks.; tomatidine, digitonin, ergosterol, cholesterol and stigmasterol from Sigma Chemical Company, Poole, Dorset; and campesterol and sitosterol from Field Instruments Ltd., Richmond, Surrey. Cholesterol-[4-^{14}C] (sp. act. 58 mCi/mmol) and sitosterol-[4-^{14}C] (sp. act. 53.6 mCi/mmol) were from the Radiochemical Centre, Amersham. PGA were extracted from dark grown potato sprouts. Frozen sprouts were homogenized and extracted in 94% MeOH acidified with 2% HOAc (2 ml/g fr. wt) for 24 hr. The slurry was Buchner-filtered and the residue extracted 2 × with 64% MeOH for a total of 24 hr. Filtrates were combined, reduced to *ca* 1/6 vol. by rotary evapn at 45° and adjusted to pH 10 with NH_4OH. After 16 hr at 4°, the extract was centrifuged at 27 000 *g* for 20 min and the pellet washed with 1% NH_3 and centrifuged as before. After evapn the pellet was dried in a $CaCl_2$ desiccator and extracted 3 × with 100 ml hot MeOH. After each extraction the residue was separated by centrifuging at 27 000 *g* for 20 min. MeOH extracts were combined and reduced to dryness *in vacuo* at 35°. The residue was exhaustively extracted with warm C_6H_6 to remove pigmentation and then taken up 3 × in hot MeOH. The MeOH was evapd off *in vacuo* at 35° and the residue dissolved in the minimum vol. of 75% EtOH at 70°. On cooling, crystals appeared which were separated by centrifuging at 16 000 *g* for 20 min. Further cooling of the supernatant at −20° yielded a second batch of crystals which were separated as previously. Both batches of crystals were combined and dissolved in 70% EtOH and re-crystallized 2 ×. After spinning down, crystals were washed with petrol (bp 40–60°) and dried; mp 280–290°, $[\alpha]_D^{EtOH}$ − 59.3°; lit. mp. 285°, $[\alpha]_D$ − 58·5° and −60° [28]. TLC on Si gel G showed tow Dragendorff positive spots when developed in 95% EtOH (R_f 0.25 and 0.48) and in BuOH–HOAc–H_2O (10:3:1) (R_f 0.08 and 0.20) which corresponded with α-solanine (authentic) and α-chaconine [29], respectively. Spraying plates with ceric ammonium sulphate reagent and heating to 105° for 30 min showed that no major organic contamination was present. Hydrolysis with N HCl resulted in the appearance of one further spot on TLC which corresponded with solanidine.

In initial experiments, steroids were prepared in 96% EtOH and mixed with sterol solns (7.5 mg/ml 96% EtOH) in the ratio of 2:0.1 ml in 15 ml polypropylene centrifuge tubes. Where radiosterols were used, 1 μCi of sterol was added to the unlabelled sterol soln before making to vol. After mixing, solns were heated at 90° for 5 min then cooled, first at room temp. for 1 hr then at 4° overnight. Tubes were spun at 27 000 *g* for 30 min and supernatants decanted. Where non-radioactive sterols were used, a 0.5 ml sample of supernatant was evapd to dryness in a stream of N_2 and taken up in 0.25 ml EtOAc containing 100 μg 5α-cholestane. A 1 μl aliquot of this sample was analysed by GLC in a glass column (2 m × 6 mm) packed with 3% OV-101 on Gas Chrom Q. N_2 flow rate was 50 ml/min, column temp. 275° and FID temp. 325°. 5α-Cholestane acted as internal standard and sterol was quantified using a pre-programmed Supergrator Mk II integrator. All sterols produced single peaks. Where radiosterols were used, a 0.5 ml sample of supernatant was added to 4 ml dioxan-based scintillation fluid (NE 250 from Nuclear Enterprises Ltd., Edinburgh) and counted on a scintillation spectrometer. Background was 28 cpm and counting efficiency 80%.

Turbidity in tomatine/sterol mixtures was measured using a Corning–Eel nephelometer. Mixtures consisted of 5 ml 1 mM tomatine plus 0.1 ml 10 mM sterol in 96% EtOH. The instrument was zeroed initially, and throughout the experiment, with a control soln containing sterol only. Full scale was set using a tube containing tomatine as above plus 0.25 ml 10 mM sitosterol which had been left for 2 hr. All tubes were agitated for 2 sec on a Whirlimixer prior to reading.

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