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SHORT COMMUNICATIONS

The protective action of glycyrrhizin against saponin toxicity

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Saponins are known to be of potential medical value, as antiinflammatory [1], antiexudative, and antiviral agents [2, 3] and even as tumor inhibitors [4–6]. Their therapeutic applications are however very limited, since when taken orally they are poorly absorbed from the intestines and when injected, especially intravenously, most of them are very toxic and induce marked hemolysis. It is therefore of great interest to find ways of inhibiting saponin-induced hemolysis.

Recently [7] some purified nonhemolytic ginseng saponins were reported to protect washed erythrocytes against the deterring effect of other hemolytic ginseng saponins. Glycyrrhizin was found to possess the same capacity against "saponin (Merck)". We wish to report the results of our investigations which show that glycyrrhizin is a general inhibitor of saponin and sapogenin hemolysis.

The *in vitro* experiments were performed on washed bovine erythrocytes (1% v/v). Isotonic phosphate buffer pH 7.4 and hemolysin (saponins and sapogenins as given in Tables 1 and 2) solutions were prepared as previously described [8]. Ammoniated glycyrrhizin (Fluka) was dissolved in buffer solution.

Inhibition of hemolysis by glycyrrhizin was determined by running two sets of experiments in parallel:

(a) The erythrocyte suspension (2 ml) was incubated at 37° with glycyrrhizin solution and buffer to give a final vol. of 3 ml. After 1 hr buffer and then hemolysin solution were added to give a final vol. of 4 ml. (In those experiments in which dimethyl sulfoxide-H_2O was the solvent for the hemolysin, this mixture was added instead of buffer to give the final vol. of 4 ml.) The mixtures were incubated at 37° for another hr (the standard time of incubation for hemolysis tests) and the percentage of hemolysis was determined by standard methods [8].

(b) Erythrocyte suspension (2 ml) was incubated with buffer (1 ml) for 1 hr at 37°, then buffer solution (or dimethyl sulfoxide-H_2O) and hemolysin were added to give a final vol. of 4 ml. The mixtures were incubated for another hr and percentage hemolysis was determined. Percentage inhibition was calculated as:

$$\left(1 - \frac{\%\text{ hemolysis with inhibitor}}{\%\text{ hemolysis without inhibitor}}\right) \times 100.$$

A plot of percentage inhibition versus glycyrrhizin concentration gave a straight line for 10–90 per cent inhibition. The protective activity, P_{50}, defined as the glycyrrhizin concentration giving 50 per cent inhibition of hemolysis, was determined from this plot and these values were reproducible to within 10 per cent of their value.

The results which are summarized in Table 1 show that glycyrrhizin is a common inhibitor for all saponins tested, although it is effective only when its concentration greatly exceeds that of the hemolysin. For each particular saponin tested there is a direct relation between the concentration of hemolysin used and the concentration of glycyrrhizin required to achieve 50 per cent protection.

Our previous investigations [8, 9] have shown that most saponins induce hemolysis only after the glycosidic bonds have been hydrolysed by appropriate membrane glycosidases to the active aglycones. We considered the possibility that the protective effect exerted by glycyrrhizin is a result of the inhibition of the membrane glycosidases. We therefore tested whether glycyrrhizin affects the hemolysis obtained by the aglycones as well. From the data in Table 2 it is obvious that sapogenin-induced hemolysis is inhibited by glycyrrhizin as effectively as that of the corresponding saponins. Moreover, protection is not confined to this group of hemolysins, but is observed for the cationic surfactant *N,N*-dimethyl-*N*-(1,1-dimethyl-2-hydroxyethyl) ammonium bromide as well.

The possibility was considered that glycyrrhizin exerts its inhibitory activity by reducing the effective concentration of hemolysin through complex formation, similar to the mechanism by which cholesterol inhibits saponin hemolysis [10, 11]. The following experiments were carried

Table 1. Glycyrrhizin inhibition of saponin induced hemolysis in washed erythrocytes

Saponin	Concentration of saponin (M)	% Hemolysis	P^*_{50} mg/ml	P_{50} (M)
digitonin	3.3×10^{-6}	50	2	2.4×10^{-3}
digitonin	2.8×10^{-6}	30	0.75	9×10^{-4}
escin	2.2×10^{-6}	90	>4	$>4.5 \times 10^{-3}$
escin	1.9×10^{-6}	65	>4	$>4.5 \times 10^{-3}$
escin	1.8×10^{-6}	50	>4	$>4.5 \times 10^{-3}$
escin	1.4×10^{-6}	32	3.75	4.5×10^{-3}
tomatin	8×10^{-6}	57	0.75	9×10^{-4}
tomatin	5×10^{-6}	30	0.56	6.7×10^{-4}
saponin A	2.7×10^{-7}	55	2.8	3.3×10^{-3}
saponin A	2.4×10^{-7}	26	1	1.2×10^{-3}

*P_{50} = the concentration of glycyrrhizin giving 50% inhibition of hemolysis.
This value was obtained from the plot of percentage inhibition versus glycyrrhizin concentration. The P_{50} values were reproducible to within 10 per cent of their value.

Table 2. Glycyrrhizin inhibition of sapogenin induced hemolysis in washed erythrocytes

Sapogenin	Concentration sapogenin (M)	% Hemolysis	P_{50} mg/ml	P_{50} (M)
digitogenin	2×10^{-4}	30	0.375	4.5×10^{-4}
tomatidine	5.8×10^{-5}	60	0.15	1.8×10^{-4}
tomatidine	5.3×10^{-5}	48	0.15	1.8×10^{-4}
sapogenin A	3.2×10^{-7}	90	>2.5	$>3.2 \times 10^{-3}$
sapogenin A	2.8×10^{-7}	57	>2.5	$>3.2 \times 10^{-3}$
sapogenin A	2.5×10^{-7}	25	1.7	2.4×10^{-3}
N,N-dimethyl-*N*-hexadecyl-*N*-(1,1-dimethyl-2-hydroxyethyl) ammonium bromide	8.2×10^{-6}	50	4.3	5×10^{-3}

out: (a) the hemolysin and glycyrrhizin were incubated for 15 min at 37° before they were added to erythrocytes, (b) first glycyrrhizin and then hemolysin were added to erythrocytes, (c) erythrocytes were preincubated for 1 hr with glycyrrhizin before the hemolysin was added. The extent of inhibition obtained in these experiments for a number of hemolysins is summarized in Table 3. The results exclude the possibility of complex formation, since preincubation of hemolysin with glycyrrhizin did not increase protection. The fact that greatest inhibition was obtained by preincubation of glycyrrhizin with the erythrocytes (experiment c) might indicate that the glycyrrhizin, which is known to be absorbed by the erythrocytes, prevents the access of the hemolysin to its receptor. This explanation would also account for the fact that glycyrrhizin is a non-specific inhibitor to the hemolysis of saponins and sapogenins as well as cationic surfactants. Moreover, the inhibition was found to be reversible, since washing of the erythrocytes which had been incubated with glycyrrhizin eliminated any inhibitory effect.

On the basis of these results it was decided to investigate the influence of glycyrrhizin on the toxicity of saponins *in vivo*. Digitonin, a commercially available saponin, was used as model hemolysin. Since it has been established that the fatal effect of saponins cannot be ascribed solely to their hemolytic activity, we investigated the effect of glycyrrhizin on two parameters of digitonin toxicity: (a) mortality rate and (b) hemolysis. Digitonin is only sparingly soluble in water and thus a cosolvent system of propylene glycol, dimethyl sulfoxide and water (2:1:2) was used. The intravenous injection of 0.1 ml of this solvent produced no toxic effects in mice weighing 22 g each (15 animals tested). The experiments were conducted at digitonin concentrations which produce 50 per cent mortality (LD_{50}) by i.v. injection of 0.05 ml digitonin solution (10.1 mg/ml) into the tail of mice weighing exactly 22 g. (LD_{50} = 23 mg/kg). For the inhibitory tests glycyrrhizin (13 mg/ml and 25 mg/ml) was dissolved in the digitonin solution to give final dosages of 30 mg/kg and 57 mg/kg when injecting 0.05 ml.* At the lower glycyrrhyzin dosage, mortality was not reduced (40 animals tested): at the higher dosage, however, a 40 per cent decrease in mortality was observed when a group of 45 animals was tested ($P < 0.01$).

The influence of glycyrrhizin on the hemolytic effect of digitonin *in vivo* was investigated at the same concentrations of saponin and glycyrrhizin as in the mortality tests. The extent of hemolysis was determined on heparinized blood collected from animals decapitated immediately after injection. Tests performed on 40 animals showed that at both dosages glycyrrhizin did not reduce hemolysis (which was about 30 per cent).

Table 3. Extent of glycyrrhizin* inhibition under various experimental conditions

Hemolysin	Concentration of hemolysin	% Inhibition in experiment (a)	(b)	(c)
digitonin	3.3×10^{-6}	45	43	55
digitonin	2.8×10^{-6}	30	31	50
tomatin	8×10^{-6}	26	25	50
escin	1.4×10^{-6}	32	35	47
tomatidine	5.3×10^{-5}	49	48	50

*The glycyrrhizin concentrations are as outlined in Tables 1 and 2.

The discrepancy between the *in vitro* and the *in vivo* inhibitory influence of glycyrrhizin against hemolysis, apparently has to be ascribed to the large excess of inhibitor needed to prevent the hemolysis. *In vitro* the ratio of inhibitor to hemolysin was 400 whereas *in vivo* the ratio could not exceed 2.5 because of the toxicity of the glycyrrhizin. On the other hand, glycyrrhizin markedly affected the second toxicity parameter investigated, i.e., mortality, although the same saponin-inhibitor ratio was used as in the hemolysis tests. These results further support former observations [12] that there need be no relationship between the lethal and the hemolytic effect of saponins.

Our results indicate that non-hemolytic saponins, which protect against *in vitro* hemolysis, might prove to be effective inhibitors to the toxic effects of saponins.

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* Toxic effects (such as convulsions, slight hemolysis etc.) occurred in animals receiving 70 mg/kg glycyrrhizin, below which, however, glycyrrhizin proved to be non toxic.

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Effect of gamma-butyrolactone and baclofen on plasma prolactin in male rats*

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Gamma-hydroxybutyrate (GHB), which occurs as a natural metabolite in mammalian brain [1], or its precursor gamma-butyrolactone (GBL), given systemically, can increase dopamine levels in the neostriatum without affecting norepinephrine or serotonin levels [2]. GHB or GBL does not inhibit monoamine oxidase or catechol-*o*-methyltransferase indicating that the increase in dopamine is not due to interference with its metabolism [2]. GHB causes a marked decrease in the utilization of dopamine [3]. Unit recordings of dopamine neurons in the zona compacta of the substantia nigra indicate that GHB administered systemically decreases the firing of these neurons [4]. These studies are consistent with the thesis that GHB inhibits impulse flow in dopamine neurons in the neostriatum [5].

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It has been proposed that the effect of GHB on dopamine neurons may be due to a direct or indirect stimulation of an inhibitory gamma-aminobutyric acid (GABA) mechanism in the substantia nigra [6].

Dopaminergic neurons of the tuberoinfundibular region of the hypothalamus inhibit prolactin release from the anterior pituitary by a direct inhibitory effect of dopamine on the pituitary [7] or by promoting the release of prolactin inhibitory factor [8], or by both mechanisms. Drugs such as dopamine receptor blockers, e.g. chlorpromazine, which decrease the dopaminergic influence on the pituitary, increase plasma prolactin [9]. Alpha-methylparatyrosine (AMPT) which inhibits the synthesis of dopamine also increases plasma prolactin [10]. Intraventricular injection of GABA raises prolactin on the morning of proestrous in intact female rats and in ovariectomized rats [11].

We were interested in determining if GHB inhibited impulse flow in the dopaminergic neurons of the hypothalamus and thereby increased plasma prolactin. We report

Table 1. Effect of drugs on rat plasma prolactin*

	Dose (mg/kg)	Duration	Plasma prolactin† (ng/ml)	*P*
Saline		30 min	10.4 ± 3.6	
Gamma-hydroxybutyrolactone	200	30 min	10.8 ± 4.5	NS‡
	400	30 min	14.4 ± 3.6	NS
	750	30 min	42.4 ± 14.6	<0.01
	1500	30 min	61.7 ± 14.6	<0.01
Baclofen	20	1 hr	9.7 ± 2.5	NS
	25	1 hr	47.2 ± 23.4	<0.01
	50	1 hr	57.6 ± 21.7	<0.01
Alpha-methylparatyrosine-methyl ester	125 × 2	4, 24 hr	33.8 ± 7.7	<0.01
Trifluoperazine	2.5	1 hr	68.4 ± 12.7	<0.01

* All groups consisted of five male rats.
† Mean ± S. D.
‡ Not significant.