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EFFECT OF ESTER GROUPS ON THE HAEMOLYTIC ACTION OF SOME SAPONINS AND SAPOGENINS

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Abstract—The saponin and sapogenin of *Styrax officinalis* L. both being benzoic acid esters, were found to be highly haemolytic, producing haemolysis at equal molar concentrations. No haemolytic activity was detectable after saponification. Methylation of the carboxylate group in the glycosidic part of the saponin, only slightly enhances activity. The haemolytic activity of some sapogenins was investigated and compared with that of their acetic and benzoic acid esters. All sapogenins were found to be highly haemolytic, esterification enhancing this property. It is proposed that the aglyconic part of the saponin molecule is the main factor in determining the haemolytic properties of saponins.

VERY little is known concerning the relationship between the chemical constitution and the haemolytic properties of saponins. Although it is known that sapogenins have slight haemolytic properties, it is generally assumed that this property, in saponins, is to a large extent due to the sugar component. This assumption led Tschesche and Wulff[1] to test the influence of the glycosidic branching in digitonin on its haemolytic properties. It was shown that the straight chain glycoside desgluco-desxylo-digitonin was still highly haemolytic although less than the parent compound. Nevertheless it was concluded that no sugar branching was necessary for haemolysis.

On the other hand the importance of the aglyconic component for the haemolytic process has been proved in aescin.[2, 3] Saponification, which removes the tiglic and acetic acid ester functions of the aglyconic part abolishes all haemolytic and pharmacological activities.

The saponin isolated from *Styrax officinalis* L. proved to be another highly haemolytic saponin ester.[4] The aim of our present investigation was to determine the role of the esteric function in haemolysis by the *Styrax* saponin as well as by other known saponins and sapogenins. Furthermore we hoped to prove which part of the saponin molecule is responsible for its haemolytic properties.

EXPERIMENTAL

Styrax saponin, which will be referred to as "saponin A", was obtained according to the method described by Wall.[5] The saponin precipitated in glistering plates after the two-phase system butanol-water had been concentrated to about one fifth of the original volume. Saponin A was found to have the following properties: m.p. 242°;

$[\alpha]_D$ in ethanol −33·3°* (found: C, 56·5; H, 7·6. $C_{58}H_{88}O_{27} \cdot H_2O$ requires C, 56·4; H, 7·3. $C_{58}H_{90}O_{27} \cdot H_2O$ requires C, 56·3; H, 7·4%).

The saponin obtained by basic hydrolysis of saponin A will be referred to as saponin B. 200 mg of saponin A were dissolved in 10 ml ethanol (50%), 1 ml of methanolic potassium hydroxide (1 N) was added and the solution heated under reflux for 2 hr. After cooling the solution was made very slightly acid with hydrochloric acid 0·5 N. All the solvent was then evaporated at room temperature in a stream of air, and the residue washed twice with small volumes of water. It was then crystallized from methanol. Saponin B was found to have the following properties: m.p. 244°; $[\alpha]_D$ in 50% ethanol −19° (found: C, 54·48; H, 7·85. $C_{51}H_{84}O_{26} \cdot H_2O$ requires C, 54·1; H, 7·61. $C_{51}H_{86}O_{26} \cdot H_2O$ requires C, 54·05; H, 7·77%).

Sapogenin A and sapogenin B, the corresponding aglycones of saponin A and saponin B, were obtained as described in a previous paper.[4]

The ammoniated glycyrrhizin, glycyrrhetinic acid, digitonin, kryptogenin, diosgenin, tigogenin and tigogenin acetate, used in this investigation are commercial products.

Digitogenin was obtained by acid hydrolysis of digitonin.[6]

The sapogenin acetates and benzoates were obtained by dissolving the sapogenin in a small volume of pyridin and adding equimolar quantities of acetic anhydride or benzoyl chloride. The mixture was left for 48 hr at room temperature and then poured into ice-cold water, filtered and crystallized to constant melting point.

Methyl esters were obtained by dissolving the acid in absolute methanol, after which an ethereal diazomethane solution was added until a pale yellow colour persisted. The mixture was left for 2 hr and then the solvent evaporated.

Glycyrrhetinic acid methyl ester was purified by crystallization from methanol.

The methyl esters of saponin A, saponin B and glycyrrhizin were purified by dissolving in methanol and precipitating by the addition of ether (no solvent system suitable for crystallization of the compounds was found).

Melting points and analyses of the esters are given in Table 1.

TABLE 1. MELTING POINTS AND ANALYSES OF THE ESTERS SYNTHESIZED

Compound	m.p. (°C)	Analysis			
		Found		Calculated	
		C%	H%	C%	H%
Tigogenin benzoate	227	78·04	9·60	78·40	9·23
Kryptogenin diacetate	188	72·30	9·30	72·37	8·95
Kryptogenin dibenzoate	183	76·54	7·79	77·10	7·83
Diosgenin acetate	200	76·05	9·50	76·31	9·64
Diosgenin benzoate	217	78·47	8·97	78·76	8·88
Glycyrrhetinic acid methyl ester	258	76·42	9·76	76·85	9·92
Acetylglycyrrhetinic acid	320	74·70	9·83	75·0	9·36

Haemolysis

Blood. Citrated rat blood was used. The erythrocytes were separated by centrifugation and washed with an isotonic buffer solution until the supernatant was colourless.

* The authors regret a printing error in Ref. 4, p. 527 line 10, which should read instead: $[\alpha]_D$ in ethanol −33·3°.

The erythrocytes were then diluted with the same buffer to give a 2 per cent suspension.

Solutions of haemolysing agents. Saponins and their methyl esters were dissolved in buffer.

All sapogenins and sapogenin esters which were insoluble in the buffer were dissolved in dimethyl sulfoxide (DMS). Care was taken that no precipitation of the solute occurred on dilution with the buffer.

The isotonic buffer solution used had the following composition:

$Na_2HPO_4 . 2H_2O$	3·95 g
KH_2PO_4	0·76 g
NaCl	7·2 g
Aqua dist.	*ad* 1000 g

The pH was adjusted to 7·4.

Haemolysis test. To 2 ml of the 2 per cent suspension of erythrocytes varying amounts of each haemolytic agent were added and the volume was made up to 4 ml with buffer. The mixture was left at 22° for 3 hr. The supernatant obtained after centrifugation was diluted with an equal volume of buffer, and the optical density was measured at 540 mμ. Complete haemolysis of a standard sample was effected by treatment with water. The H_{50} value (i.e. the concentration giving 50 per cent haemolysis) was determined by plotting per cent haemolysis against concentration.

The tolerance of erythrocytes towards dimethyl sulfoxide was tested and found to be 0·3 ml/ml test solution.

The effect of this concentration of DMS on saponin A and digitonin haemolysis was tested and neither an accelerating nor an inhibiting effect was found.[7]

Surface tensions of aqueous saponin solutions were determined by a Noüy tensiometer. The concentrations of the solutions tested were 200 μg/ml. The results are given in Table 2.

TABLE 2. SURFACE TENSION OF AQUEOUS SAPONIN SOLUTIONS

Saponin 200 (μg/ml)	Surface tension (dyn/cm²)
Water	65·8
Saponin A	57·3
Saponin A methyl ester	49·5
Saponin B	64·3
Saponin B methyl ester	61·6
Glycyrrhizin	50·0
Glycyrrhizin methyl ester	58·0
Digitonin	48·8

RESULTS

The haemolytic potency of the compounds tested is expressed by their H_{50} values.

Saponin haemolysis

The large number of hydroxylic groups present in the sugar part of the saponin, precludes the selective esterification of the aglyconic hydroxyls. Data relating haemolytic properties to ester function were therefore investigated by us only for *Styrax* saponin, i.e. saponin A, which is a naturally occurring saponin ester and for

glycyrrhizin, in which the ester function was introduced by methylation of the carboxylic acid groups. This methyl ester could not be crystallized for analysis, but the presence of an ester function was established by a positive hydroxamic acid test.[8] In this compound it must be assumed that more than one carboxylic acid group underwent methylation.

The effect of haemolysis of methylating a carboxylate group of the sugar part, was tested for saponin A, which has a glucuronic acid residue.

The results of all the above experiments on saponin haemolysis are summarized in Table 3. They show that while the basic hydrolysis of saponin A to the unesterified

TABLE 3. HAEMOLYTIC POTENCY (EXPRESSED AS H_{50}) OF SAPONINS AND THEIR ESTERIFIED DERIVATIVES

Saponin	H_{50} (μg/ml)
Saponin A	1·42
Saponin A methyl ester	0·85
Saponin B	>1000
Saponin B methyl ester	>1000
Glycyrrhizin	>2500
Glycyrrhizin methyl ester	2150
Digitonin	3·6

saponin B completely abolishes all haemolytic activity, the methylation of the carboxylate group in saponin A slightly increases it. The methyl ester of glycyrrhizin was found to be slightly haemolytic in contrast to the parent compound which is non-haemolytic.

The esterified saponins from *Styrax* possess higher surface activity than the hydroxylic compound. In glycyrrhizin however esterification reduces surface activity. Comparison of Tables 2 and 3 shows no correlation between surface activity and haemolytic activity.

Sapogenin haemolysis

The H_{50} values for various sapogenins and for their acetic and benzoic acid esters are summarized in Table 4. It will be seen that all sapogenins tested were haemolytic, most of them in small concentrations. In all cases the introduction of an ester group into the molecule enhances haemolysis. This is most pronounced in the cases of sapogenin A (as compared with sapogenin B), and glycyrrhetinic acid (as compared with glycyrrhetinic acid methyl ester and acetyl glycyrrhetinic acid).

The H_{50} values of acetic and benzoic acid esters show no significant differences.

DISCUSSION

Owing to the scarcity of pure commercial saponins, our investigations had to be conducted mainly on sapogenins, and only in few cases, i.e. digitonin, saponin A and glycyrrhizin, was it possible to compare the haemolytic potency of the saponins and their respective sapogenins. Nevertheless, from the results obtained it appears that the haemolytic effect of the saponin is to be ascribed mainly to the aglyconic component. Only by this assumption can we explain the fact that all sapogenins tested were highly haemolytic.

In the three cases where the activity of the saponin and sapogenin could be compared it was seen that in saponin A the glycoside and the aglycone gave 50 per cent haemolysis at exactly equal molar concentrations, in glycyrrhizin only the aglycone possessed haemolytic acitivity, but in digitonin the glycoside was more active.

TABLE 4. HAEMOLYTIC POTENCY (EXPRESSED AS H_{50}) OF SAPOGENINS AND THEIR ESTERS

Sapogenin	H_{50} (μg/ml)
Sapogenin A	0·7
Sapogenin B	>3
Glycyrrhetinic acid	260
Glycyrrhetinic acid methyl ester	63
Acetyl glycyrrhetinic acid	33·5
Tigogenin	48
Tigogenin acetate	37
Tigogenin benzoate	40
Diosgenin	52
Diosgenin acetate	42
Diosgenin benzoate	45
Kryptogenin	134
Kryptogenin diacetate	87
Kryptogenin dibenzoate	36
Digitogenin	27

Undoubtedly an ester group in the sugar part of a saponin slightly enhances haemolysis (saponin A methyl ester is twice as potent as saponin A, and the methyl of aescin was found to be 2·5 times that of aescin[9]). However, the presence ester of the ester group in the aglyconic residue is indispensable for haemolysis in both these saponins.[3]

It might be argued that the greater influence on haemolysis of the ester group in the aglycone as compared with that in the sugar part is due to the difference in the groups esterified, i.e. hydroxylic or carboxylic. But from the results obtained for glycyrrhetinic acid and its methyl and acetyl derivatives it seems that the nature of the ester is of minor importance. The comparison of the H_{50} values of glycyrrhetinic acid, glycyrrhetinic acid methyl ester and acetyl glycyrrhetinic acid (Table 4) show that the acetate is eight timesmore potent than the parent compound but only twice as active as the methyl ester.

No explanation can be given for the fact that ester groups in saponins as well as in sapogenins enhance haemolytic potency, and that the absence of these groups may even abolish this property completely. Although saponin B has only a slight surface activity as compared with saponin A, it seems that the haemolytic activity of saponin A cannot be ascribed to surface phenomena, as in saponin A equal molar concentrations of saponin and sapogenin cause equal haemolysis. Therefore a complex formation with one of the erythrocyte membrane constituents must be assumed. For all sapogenins which lack surface activity this same mechanism must be assumed. It seems therefore that either the presence of an ester group or the absence of a polar group enhances the formation of this complex.

The nature of the complex formed, and the effect of various esters in sapogenins on haemolysis are now under investigation.

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