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ON THE SIMILARITY OF HEMOLYSIS INDUCED BY PLANT SAPOGENINS AND BY NEUTRAL STEROIDS

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Abstract—The hemolytic properties of a variety of neutral steroids has been investigated. Great similarity could be observed in their structural requirements as compared with those of the sapogenins. The following common factors were observed: esterification of an —OH group, or its oxidation to the respective ketone always enhances hemolysis. Hemolysis is only slightly influenced by either the A/B ring junction, or by the stereochemistry of the 3-substituent. Nevertheless the preferred structures for hemolysis are: 5α > 5β; 3α—OH > 3β—OH; 3β—acetoxy > 3α—acetoxy. It is suggested that sapogenin hemolysis should not be treated as a phenomenon specific to these compounds, since the same type of hemolysis is induced by a great variety of other compounds.

PREVIOUS investigations[1–3] on the hemolytic effect of plant sapogenins indicated that the extent of this property is hardly influenced by the specific structure of the hemolysing agent, i.e. the same effects are obtained by triterpens, spirostanols and kryptogenin.

On the other hand it has been proved that the presence of strong polar groups—hydroxyl or carboxylic acid—always diminishes the hemolytic capacity of the sapogenins, while esterification of these groups causes manifold enhancement of this property. These findings hold good for sapogenins of whatever type tested. The position of the polar groups in the molecule, however, influences the intensity of hemolysis. This has been shown for the triterpens by Schlösser and Wulff[4] and it is probably a characteristic common to all sapogenins.

For some time, the existence of hemolytic steroids, other than bile acids, has been known.[5–7] Weissmann and Keiser[8] showed that some neutral steroids were strongly hemolytic and that acetylation of hydroxyl functions in these compounds enhanced this property.

These findings, together with the observation that the hemolytic properties of sapogenins cannot be ascribed to a specific chemical structure, induced us to investigate the possible existence of a common factor in sapogenins and neutral steroids to which hemolysis may be ascribed.

EXPERIMENTAL

The steroids used were commercial products obtained as a gift from Ikapharm Pharmaceutical Laboratories.

Acetylations were performed as usual (pyridin–acetic anhydride). The acetates obtained were crystallized and their purity and authenticity were tested by thin layer chromatography and i.r. spectra.

β-Amyrin and epi-β-Amyrin were a gift from Dr. R. Ikan, Department of Organic Chemistry, the Hebrew University, Jerusalem.

The steroidal sapogenins used were all commercial products.

Hemolysis

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 erythrocytes were then diluted with the same buffer to give a 1% suspension.

Solutions of hemolysing agents

All compounds were dissolved in dimethyl sulfoxide (DMSO). The solutions were then tested to assure that no precipitation occurred on dilution with water and when necessary, the concentration of the DMSO solutions was reduced until no precipitation occurred on dilution. Before use in the hemolysis test the DMSO solutions were diluted with distilled water in the proportion of DMSO solution–water 5:1. (The dilution of the DMSO prior to its addition to the erythrocyte suspension eliminates the hemolytic effect of this solvent almost completely.)

Hemolysis test

The test solution consisted of 1·4 ml erythrocyte-suspension, varying volumes of the above DMSO–water solutions of the hemolysing agent and varying volumes of DMSO water mixture (5:1) to make up the volume to 2 ml. The components were added in the following order: first the erythrocyte suspension then the DMSO–water mixture and lastly the hemolysing agent. The mixture was incubated for 2 hr at 37°, then centrifuged at 1500 rev/min for 10 min and the optical density of the supernatant determined at 540 nm. Complete hemolysis of a standard sample was effected by treatment with styrax saponin. The H_{50} values (the concentration giving 50 per cent hemolysis) were determined by plotting percentage hemolysis against concentration.

RESULTS

Table 1 contains the data on the hemolytic capacity of a variety of neutral steroids. Most of them are highly hemolytic to an extent comparable to that of steroidal sapogenins, Table 2 and Table 2 of Ref. 2.* The results obtained emphasize the great similarities between the factors influencing hemolysis both in sapogenins and in neutral steroids.

The effect of esterification

The most striking among the similarities is the enhancing effect which esterification has on the hemolytic capacity, which in certain cases is 10-fold and more (oleanolic acid $H_{50} > 2 \times 10^{-4}$ M compared with oleanolic acid acetate $H_{50} = 1{\cdot}2 \times 10^{-5}$ M;[2] and 5α-pregnane-3β-ol-20-one, compound 28, $H_{50} = 1{\cdot}2 \times 10^{-4}$ M, compared with the corresponding acetate, compound 29, $H_{50} = 6{\cdot}7 \times 10^{-6}$ M). The enhancement of hemolysis by esterification of one polar group in the hemolysin has been investigated most thoroughly by us and was confirmed in *all* compounds tested—

* The discrepancy between the H_{50} values in Table 2 and in Table 4 Ref. 1 is to be ascribed to the fact that the previous experiments were performed at 22° while the present tests were conducted at 37°.

sapogenins as well as neutral steroids. The extent of increase in the hemolytic capacity which esterification has depends, however, on the position of the hydroxyl function in the molecule, as we shall endeavour to elaborate more fully in a future publication.

The effect of the carbonyl group

Oxidation of a hydroxyl group to a carbonyl function has an enhancing effect on the hemolytic potency of sapogenins as well as of neutral steroids very similar to that

TABLE 1. HEMOLYTIC POTENCY (EXPRESSED AS H_{50}) OF NEUTRAL STEROIDS AND THEIR ACETATES

Number of compound	Common name	Chemical name	H_{50} expressed as molar concentration $\times 10^5$
1	Androsterone	3α-hydroxy-5α-androstane-17-one	28
2	Androsterone acetate		7·2
3	epi-Androsterone	3β-hydroxy-5α-androstane-17-one	37
4	epi-Androsterone acetate		4·2
5	Ethiocholanolone	3α-hydroxy-5β-androstane-17-one	43×10^{-5} M gives 30%
6	Ethiocholanolone acetate		8·4
7	Dehydroisoandrosterone	3β-hydroxy-5-androstane-17-one	38
8	Dehydroisoandrosterone acetate		8·8
9	Dehydroisoandrosterone sulfate, sodium salt		>30
10	Androstanolone	17β-hydroxy-5α-androstane-3-one	40
11	Androstanolone acetate		2·2
12	5β-dihydrotestosterone	17β-hydroxy-5β-androstane-3-one	>80
13		3α, 17β-dihydroxy-5α-androstane	11×10^{-5} M gives 10%
14		3α, 17β-dihydroxy-5α-diacetate	1·4
15		5α-androstane-3-one	0·9
16		5α-androstane-17-one	0·3
17		5α-androstane	5×10^{-5} M gives 20%
18		5β-androstane	5×10^{-5} M gives 30%
19		testosterone	>90
20		testosterone acetate	9
21		3β-hydroxy-5α-androstane	1·4
22		3β-hydroxy-5α-androstane acetate	0·3
23		17β-hydroxy-5α-androstane	2·0
24		17β-hydroxy-5α-androstane acetate	0·07
25		5α-androstane-17-one	0·3
26		3α-hydroxy-5α-pregnane-20-one	>3·9
27		3α-hydroxy-5α-pregnane-20-one acetate	1·1
28		3β-hydroxy-5α-pregnane-20-one	12
29		3β-hydroxy-5α-pregnane-20-one acetate	0·67
30		3β-hydroxy-5β-pregnane-20-one	20
31		3β-hydroxy-5β-pregnane-20-one acetate	1·5
32		3α-hydroxy-5β-pregnane-20-one	16
33		3α-hydroxy-5β-pregnene-20-one acetate	2·2
34	Pregnenolone acetate	3β-acetoxy-5-pregnene-20-one	2·4
35	Pregnenolone sulfate, sodium salt		>30
36	Progesterone	4-Pregnene-3,20-dione	61

of esterification, although to a lower extent. The fact is demonstrated by the following examples: compounds 21, 22, 15; 23, 24, 25; from the series of neutral steroids, and 38, 39, 40; from the series of steroidal sapogenins. However the total elimination of this function by reduction to the respective desoxy compound yields sapogenins and steroids having a lower hemolytic activity than their parent compounds as is shown by the following group of compounds: 17, 21, 23; 38, 41; and 42, 45; this applies only to those cases lacking an additional polar function, since 5α-androstane-3β-ol (No. 21) and 5α-androstane-17β-ol (No. 23) are both far more active than the corresponding diol (No. 13); and 5α-androstane-17-one (No. 16) is far more active than either 3α-hydroxy-5α-androstane-17-one (No. 1) or 3β-hydroxy-5α-androstane-17-one (No. 3).

TABLE 2. HEMOLYTIC POTENCY (EXPRESSED AS H_{50}) OF SAPOGENINS AND THEIR ACETATES

Number of compound	Common name	Chemical name	H_{50} expressed as molar concentration $\times 10^5$
38	Smilagenin	5β, 20α, 22α, 25α-spirostan-3β-ol	3·9
39	Smilagenin acetate		0·5
40	3-dehydro smilagenin		0·8
41	3-desoxy smilagenin		4·4
42	Tigogenin	5α, 20α, 22α, 25α-spirostan-3β-ol	2·5
43	Tigogenin acetate		1·4
44	3-dehydro tigogenin		insoluble
45	3-desoxy tigogenin		3
46	Diosgenin	Δ^5, 20α, 22α, 25α-spirostan-3β-ol	2·7
47	Diosgenin acetate		2·0
48	β-amyrin		2·8
49	epi-β-amyrin		2·0

The effect of the A/B ring junction

A comparison of the activity of the following pairs of compounds: 10 with 12; 1 with 5; 28 with 30; 17 with 18; and 42 with 38; indicates that the type of ring junction has only a minor influence on the hemolytic capacity of the compounds although the A/B *trans* configuration imparts to both types a higher activity than the A/B *cis* junction. As yet the effect which a Δ 5 double bond has on hemolysis has only been tested in the few compounds at our disposal. It seems however that the activity of these compounds lies within the range of the above mentioned types.

Effect of the stereochemistry of substituents at position 3

The stereochemistry of these substituents has a relatively small effect on the activity of the model compounds, but nevertheless in all the neutral steroids tested as well as in β-amyrin, the 3α—OH configuration imparts greater activity than the 3β-isomer as can be seen from the following examples: 1 > 3; 32 > 30; 49 > 48. In the respective acetates, on the other hand, the stereochemistry also has a constant effect—but here the 3β-configuration is more active than the 3α e.g. 4 > 1; 29 > 27; 31 > 33. The acetates of β-amyrin and epi-β-amyrin could not be tested because of their extremely low solubility in dimethyl sulfoxide.

DISCUSSION

In the course of these studies the striking similarity between the hemolytic properties of sapogenins and of neutral steroids has been shown. In both groups the capacity of the hemolysing agent is always influenced by its definite structure and specific functional groups in the same direction—either amplificatory or inhibitory.

Our previous assertion[2] that esterification enhances hemolysis because it eliminates a strong polar group has been reconfirmed, both in a positive as well as in a negative manner; the strong polar sodium salts of the sulfate esters (compounds 9 and 35), possess no (or very low) hemolytic properties, while oxidation of a hydroxyl to a carbonyl group markedly increases hemolysis. However it appears that the complete absence of polar groups from the hemolysin results in a degree of hemolysis lower even than that obtained by the hydroxy derivative.

Our results do not always conform with those obtained by Weissmann *et al.*[8] but both investigations show unequivocally that esterification always enhances the hemolysis by steroids. This effect is a very marked one and can therefore be observed under whatever experimental methods applied. Our results differ however with regard to the minor influence which the A/B ring junction and stereochemistry of the substituent at position 3 may have. It seems plausible that these discrepancies can be ascribed to various differences in the techniques used: in our experiments the concentrations giving 50 per cent hemolysis were compared while Weissmann *et al.* compared the extent of hemolysis at a fixed concentration of 5×10^{-4} M. Moreover, Weissmann *et al.* used alcoholic solutions which were added to the aqueous test solutions and probably part of the compounds precipitated from their solutions at this stage, thus reducing the effective concentration of the material tested.

It may be deduced that the basic structure of the hemolysin has a far smaller influence on the hemolytic potency as compared to that of the polar functional groups. In other words, provided the number and nature of the polar groups are identical, very similar activities are obtained for androstane derivatives, steroidal sapogenins and pentacyclic triterpens.

It may therefore be concluded that sapogenin and steroid hemolysis should not be treated as separate phenomena, but as one property common to a wide variety of steroids and triterpens.

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REFERENCES

1. R. Segal, M. Mansour and D. V. Zaitschek, *Biochem. Pharmac.* **15**, 1411 (1966).
2. R. Segal, I. Milo-Goldzweig, H. Schupper and D. V. Zaitschek, *Biochem. Pharmac.* **19**, 2501 (1970).
3. R. Segal, *Israel J. Chem.* in press.
4. E. Schlösser and G. Wulff, *Z. Naturforsch.* **246**, 1284 (1969).
5. I. Tateno and E. D. Kilbourne, *Proc. Soc. exp. Biol. N.Y.* **86**, 168 (1954).
6. R. H. Palmer, *Nature, Lond.* **201**, 1134 (1964).
7. A. Karpas and R. H. Palmer, *Pharmac. Rev.* **15**, 123 (1963).
8. Weissmann and H. Keiser, *Biochem. Pharmac.* **14**, 537 (1965).