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HEMOLYSIS AND AUGMENTATION OF HEMOLYSIS BY NEUTRAL STEROIDS AND BILE ACIDS*

GERALD WEISSMANN† and HAROLD KEISER

Department of Medicine, New York University School of Medicine, New York, N.Y., U.S.A.

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Abstract—Thirty-five neutral steroids and bile acids have been studied for their ability to lyse rabbit erythrocytes. Hemolytic activity was found to be associated with the 5-β-H configuration. A few Δ-4,5 steroids were active, but 5-α-H compounds were inactive. Oxygenation at C-11 or the presence of an α-hydroxyl group at C-17 abolished activity. The hemolysis induced by neutral steroids was characterized by (1) sharp concentration dependence above a critical concentration; (2) marked loss of activity at 24° compared with 37°; (3) inhibition by serum; (4) prelytic loss of potassium; and (5) marked augmentation by inactive steroids, including the 3-β-OH and 5-α-H or Δ-4 compounds which inhibit bile acid-induced hemolysis.

These results are compatible with the hypothesis that neutral steroids exert their pharmacologic effects, at least in part, through actions on the membranes of cells or their organelles.

PREVIOUS communications from this laboratory have indicated that neutral steroids and bile acids affect the release of acid hydrolases from lysosomes in granular fractions of rabbit liver and leukocytes.[1] This property of steroids is structurally specific and may be related to their general disruptive effect on biological membranes, as has been found to be the case with agents such as u.v. irradiation,[2] lysolecithin,[3] vitamin A,[3] and the streptolysins.[4] To examine this hypothesis, 35 steroid compounds‡ have been tested for their ability to cause hemolysis of rabbit erythrocytes.

Both the hemolytic properties of the bile acids and the inhibition of bile acid hemolysis by cholesterol and structurally related steroids have been thoroughly investigated;[5, 6] however, there have been few studies of the possible hemolytic effects of neutral steroids. Tateno and Kilbourne[7] found that stilbesterol and progesterone had strong hemolytic action and that desoxycorticosterone acetate was moderately hemolytic, whereas "testosterone, estradiol, cortisone, and compounds A, B, and F" were essentially inactive. Palmer,[6] too, found progesterone to be hemolytic; hemolysis was also induced by etiocholanolone, androsterone, 11-ketopregnanolone, and testosterone.

The simultaneous addition of nonhemolytic corticosteroids was found by Tateno and Kilbourne to accelerate hemolysis induced by stilbesterol.[7] In direct contrast, Berliner and Schoenheimer showed that the addition of cholesterol and structurally related compounds inhibited hemolysis caused by the bile acids.[5] Experiments to be reported below indicate that augmentation of neutral steroid hemolysis is not a

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† Senior Investigator, Arthritis and Rheumatism Foundation.

‡ For purposes of simplicity of expression, diethylstilbesterol is here referred to as a steroid.

specific property of the corticosteroids and that nonhemolytic steroids, with structural specificity required for inhibition of bile acid hemolysis, *augment* rather than inhibit hemolysis induced by neutral steroids.

MATERIALS AND METHODS

Materials. The steroid compounds were obtained from Steraloids, Inc.; their purity was checked by melting-point determination and chromotography in two solvent systems. The three bile acid compounds and 11-ketopregnanolone were the gift of Dr. Attallah Kappas. All compounds except pregnanediol were dissolved in absolute ethanol so that the final 1 to 50 dilution into the test system resulted in a concentration of 5×10^{-4} M; pregnanediol could be dissolved in ethanol only to a final concentration of $2{\cdot}5 \times 10^{-4}$ M. The required amounts of cortisone acetate, cortisol acetate and lithocholic and allocholanic acids (neutralized with NaOH) were insoluble in ethanol and were used as suspensions.

Ethanol itself is hemolytic. However, hemolysis induced by each agent was expressed as a percentage of that induced by ethanol controls. In this, as in previous studies employing ethanol or methanol, it is impossible to determine the role of sublytic concentrations of solvent in augmenting steroid hemolysis. Data obtained from K^+-leakage experiments (see below) suggest that ethanol at the concentration used did not appreciably induce sublytic ion release.

Erythrocyte suspension. Blood was obtained by cardiac puncture with heparinized syringes from young male rabbits and was used immediately. The cells were washed four times with a 0·9% NaCl solution buffered to pH 7·2 by 0·01 M phosphate buffer and finally suspended in this solution to a 10% (v/v) concentration.

Method. The procedure used was essentially that of Tateno and Kilbourne.[7] Ethanol solutions of the test compound(s) were added in 0·05 ml amounts to 2·0 ml of phosphate-buffered saline. Finally, 0·5 ml of red cells was added, and the test suspension was incubated, usually for 3 hr, at 37°. Control tubes for assessment of 100% hemolysis contained 0·5 ml of red cell suspension in 2·0 ml distilled water. After incubation, the test suspensions were centrifuged for 4 min and the supernates removed; 1·0 ml of supernate was then added to 4·0 ml of 0·6% ammonium hydroxide and the quantity of hemoglobin determined by measurement of the absorbance at 540 mμ in the Beckman DB spectrophotometer. Potassium was determined by flame photometry.

Results have been expressed as per cent of total hemolysis found with distilled water. In Table 1, results are also expressed as per cent control, i.e. (optical density at 540 mμ of sample × 100)/(optical density at 540 mμ of control). Since control hemolysis varied from 0·4% to 1·4%, these two expressions of results do not necessarily give precisely comparable figures in every case. Statistical significance was determined by a modification of the Mann-Whitney-Wilcoxon Test.[8]

RESULTS

Hemolytic effect of steroids

The hemolytic capacity of 32 neutral steroids and 3 bile acids is indicated in Table 1; this is listed in order of the per cent hemolysis induced by each agent at 5×10^{-4} M; incubation was at 37° for 3 hr. Of the compounds listed, the first eleven, the thirteenth, and the sixteenth caused significant hemolysis; the statistical significance of the difference in per cent hemolysis between the ethanol controls and allocholanic acid

TABLE 1. HEMOLYTIC ACTIVITY OF STEROIDS

Common name	Chemical name	No. of experiments	Per cent hemolysis[b]	Per cent control[c]	P-Value[d]
Ethanol[a]	Ethanol	26	0·85	100	
1. Stilbestrol	Diethylstilbestrol	17	67	6,500	<0·01
2. Lithocholic acid	5-β-cholanic acid-3-α-ol	6	27	3,280	<0·01
3. Pregnanolone acetate	5-β-pregnan-3-α-ol-20-one acetate	10	19	2,080	<0·01
4. Epipregnanolone acetate	5-β-pregnan-3-β-ol-20-one acetate	7	10	1,010	<0·01
5. Epipregnanolone	5-β-pregnan-3-β-ol-20-one	9	9·2	906	<0·01
6. Taurolithocholic acid	5-β-cholanic acid-3-α-ol-24-taurine	6	5·4	570	<0·01
7. Pregnanolone	5-β-pregnan-3-α-ol-20-one	13	4·3	511	<0·01
8. Etiocholanolone	5-β-androstan-3-α-ol-17-one	12	4·0	415	<0·01
9. Dehydroepiandrosterone acetate	5-androsten-3-β-ol-17-one acetate	8	3·8	359	<0·01
10. Pregnanedione	5-β-pregnan-3,20-dione	10	2·8	260	<0·01
11. Desoxycorticosterone	4-pregnen-21-ol-3,20-dione	6	2·7	273	<0·01
12. Allocholanic acid	5-α-cholanic acid-3-α-ol	6	2·3	270	<0·02
13. Progesterone	4-pregnen-3,20-dione	12	2·2	268	<0·01
14. 11-Ketopregnanolone	5-β-pregnan-3-α-ol-11,20-dione	6	2·2	248	<0·02
15. Allopregnanedione	5-α-pregnane-3,20-dione	6	1·9	189	<0·02
16. Dehydroepiandrosterone	5-androsten-3-β-ol-17-one	9	1·8	179	<0·01
17. 17-β-Hydroxyestradiol	1,3,5(10-estrien-3,17-β-diol	7	1·2	137	>0·10
18. Androsterone	5-α-androstan-3α-ol-17-one	9	1·2	115	>0·10
19. Testosterone	4-androsten-17-β-ol-3-one	9	1·1	144	>0·10
20. Corticosterone	4-pregnen-11-β-21-diol-3,20-dione	6	1·1	133	>0·10
21. 11-β-Hydroxypregnanolone	5-β-pregnan-3-α-11-β-diol-20-one	6	1·1	129	>0·10
22. Pregnanediol	5-β-pregnan-3-α-20-α-diol	11	1·1	126	>0·10
23. Dehydroepiandrosterone sulfate	5-androsten-3-β-ol-17-one sodium sulfate $2HO_2$	6	1·0	117	>0·10
24. Cortisol	4-pregnen-11-β-17-α-21-triol-3,20-dione	12	0·99	109	>0·10
25. 5-α-pregnanediol	5-pregnen-3-α-20-α-diol	7	0·97	141	>0·10
26. Allopregnanolone	5-α-pregnan-3-α-ol-20-one	12	0·97	108	>0·10
27. 11-Desoxycortisol	4-pregnen-17-α-21-diol-3,20-dione	9	0·93	108	>0·10
28. 11-Ketoetiocholanolone	5-β-androstan-3-α-ol-11,17-dione	6	0·92	106	>0·10
29. Cortisol acetate	4-pregnen-11-β-17-α-21-triol-3,20-dione acetate	9	0·92	109	>0·10
30. Cortisone acetate	4-pregnen-17-β-21-diol,3,11,20-trione acetate	9	0·91	104	>0·10
31. Cortisone	4-pregnen-17-β-21-diol-3,11,20-trione	13	0·88	102	>0·10
32. Epiandrosterone	5-α-androstan-3-β-ol-17-one	10	0·86	108	>0·10
33. Prednisolone[e]	1,4-pregnadien-11-β-17-α-21-triol-3,20-dione	8	0·85	115	>0·10
34. 11-β-Hydroxyetiocholanolone	5-β-androstan-3α-11-β-diol-17-one	2	0·75	100	
35. Androstenedione	4-androstan 3,17-dione	12	0·75	93	>0·10

[a] A compounds, except where otherwise indicated, were added in 0·05 ml ethanol to give a final concentration of $2·5 \times 10^{-4}$ M in 2·5 ml of a 10% suspension of rabbit erythrocytes in buffered saline. Incubaton was for 3 hr at 37°. Hemoglobin in supernates was determined by absorbance at 540 mμ.

[b] Per cent of hemolysis in 10% suspension of rabbit erythrocytes in distilled water.

[c] Absorbance of sample × 100/absorbance of ethanol control.

[d] Based on the Wilcoxon test.

[e] Added in 0·10 ml ethanol to give a final concentration of 5×10^{-4} M.

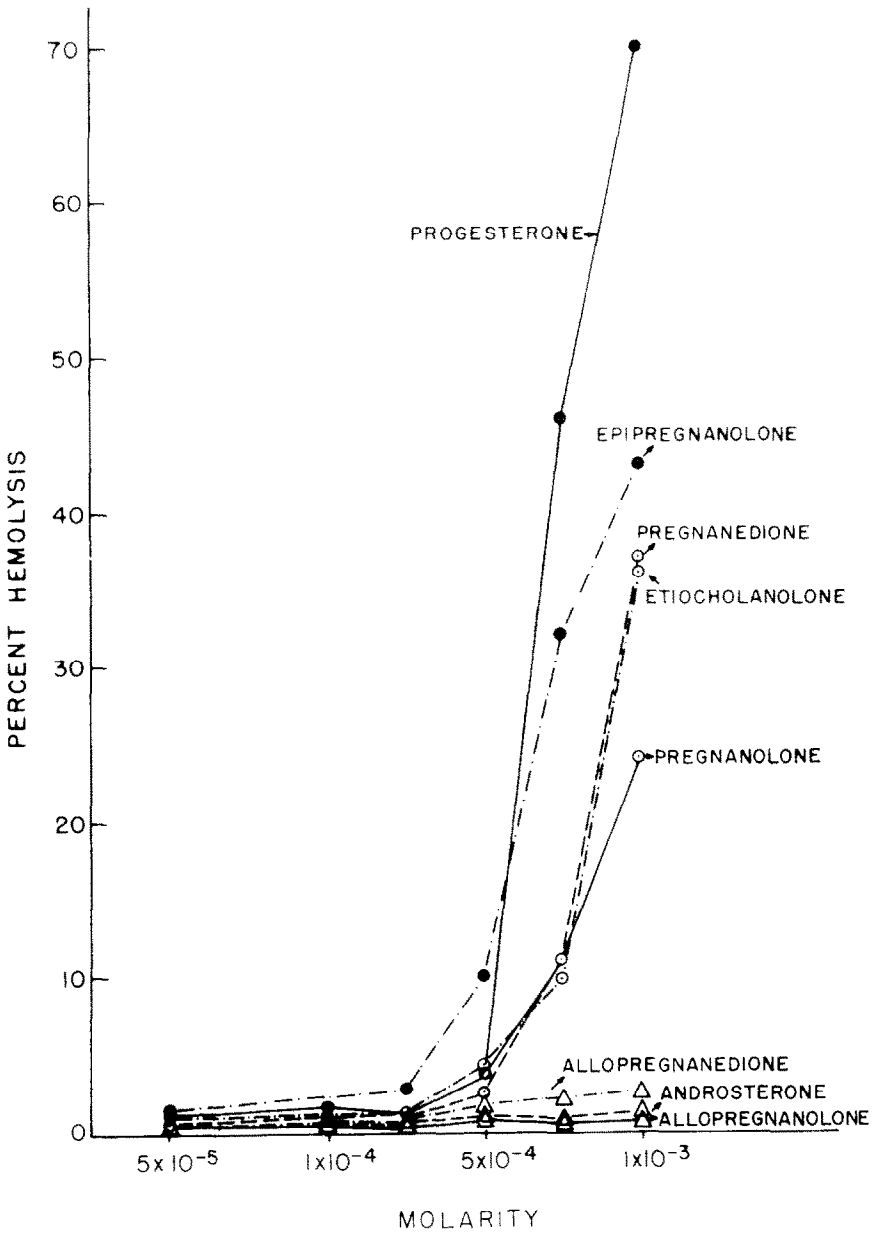

FIG. 1. Effect of concentration on hemolysis by steroids. Steroids (in ethanol) were added to 2·0 ml buffered saline (see text) together with 0·5 ml rabbit erythrocytes. After incubation at 37° for 3 hr, the hemoglobin released into the suspending medium was determined by the absorbance at 540 mμ. Results are expressed as per cent hemolysis of an equivalent erhthrocyte suspension in distilled water.

(no. 12), allopregnanedione (no. 15), and 11-ketopregnanolone is of a lower order. All other compounds were nonhemolytic.

The property of lysing rabbit erythrocytes was associated with the 5-β-H configuration, where the A : B ring junction is *cis* or highly angulated; 5-α-H compounds,

where the A : B ring junction is *trans* or planar, were relatively inactive (cf. nos. 7 and 26, 8 and 18, 10 and 15). Acetates of "active" compounds showed increased hemolytic activity (cf. nos. 3 and 7, 4 and 5, 9 and 16), whereas the presence of an oxygen at C-11 caused the loss of hemolytic activity (nos. 8, and either 28 or 34; or 11 vs. 20) as did the presence of an α-hydroxyl group instead of a ketone at C-17 (nos. 11 vs. 27). The configuration at C-3 did not seem crucial. Epipregnanolone acetate (3-β-ol) was less active than pregnanolone acetate (3-α-ol) but with the free alcohols the order of activity was reversed. This was in contrast to the requirement of the 3-α-ol,5-β-H configuration for maximal activity in the bile acid series.[5] Nor did substitutions in the C-20 or C-21 position appear crucial.

Effect of concentration

As may be seen in Fig. 1, the extent of hemolysis was dependent on the concentration of steroid present, but only over a narrow range. Most of the steroids considered hemolytic were inactive at $2{\cdot}5 \times 10^{-4}$ M and below, weakly active at 5×10^{-4} M, and increased markedly in hemolytic capacity above that concentration. Progesterone was among the weakest hemolytic compounds at 5×10^{-4} M but among the most hemolytic at $7{\cdot}5 \times 10^{-4}$ and 1×10^{-3} M. The 5-α-H steroids, and other compounds considered inactive, did not cause comparable increases in hemolysis at higher concentrations.

Effect of temperature

The extent of hemolysis induced by the most active steroids was found to be markedly temperature dependent (Fig. 2). At 5×10^{-4} M, compounds which were strongly hemolytic at 37° became far less hemolytic at 24°, with relatively little further loss of activity at 4°. The sharp increase in hemolysis which resulted from raising the concentration of active steroid from 5×10^{-4} M to 1×10^{-3} M at 37° was abolished by incubation at 4°; instead, a smaller increase in hemolytic activity with rises in concentration was found at the lower temperature. Even at 5×10^{-4} M, there was a significant increase in hemolysis caused by the active compounds at 4°, compared to the ethanol control.

Rate of potassium release and hemolysis

The extent to which hemoglobin was released during an experiment appeared to vary with each specific "active" compound studied (Fig. 3). Stilbestrol-induced hemolysis was relatively rapid during the first hour, but slowed considerably during the final hour, although only 60 to 70% of total hemolysis was achieved. In contrast, pregnanolone acetate-induced hemolysis increased in almost linear fasihon over the 3-hr incubation period. With progesterone and etiocholanolone, there was little more release of hemoglobin during the first hour than from the ethanol control. Each of the active steroids caused "prelytic" loss of potassium, i.e. release of potassium from the erythrocytes before release of hemoglobin, but they did not behave in a uniform manner. In the presence of stilbestrol and progesterone, virtually all intracellular potassium was lost by 90 min. Stilbestrol had, however, released 80%, and progesterone only 6% of total hemoglobin by that time. Potassium was released by both etiocholanolone and pregnanolone acetate with a similar, relatively slow, rate of increase. However, whereas no hemoglobin was released by etiocholanolone during

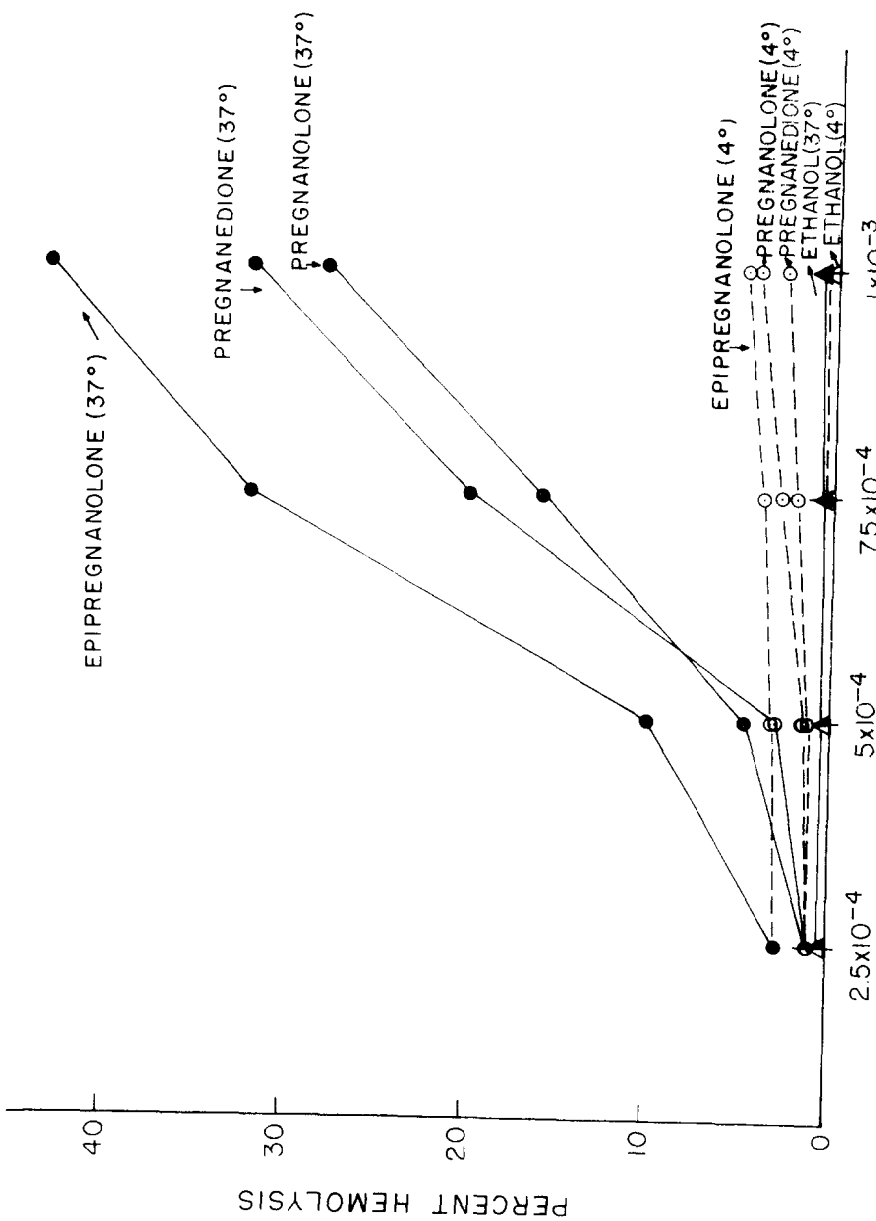

FIG. 2. Effect of temperature on hemolysis by steroids. Steroids (in ethanol) were added at $5 \cdot 10^{-4}$ M to erythrocytes for 3 hr at 37° or 4°C. Hemolysis expressed as in Fig. 1.

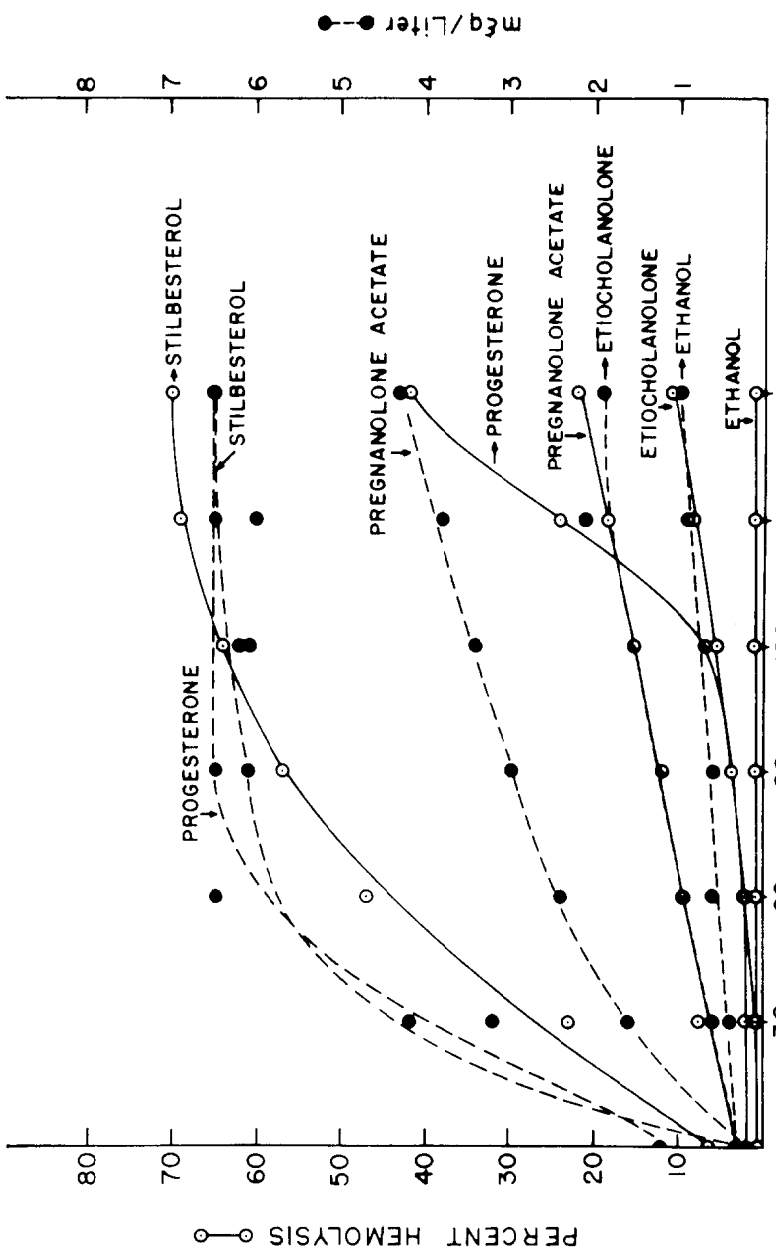

FIG. 3. Hemolysis and release of potassium by steroids. Per cent hemolysis (solid lines) and potassium concentration of supernatants in mEq/liter (broken lines) of erythrocyte suspensions incubated for times indicated at 37°. Stilbestrol and pregnanolone at 5×10^{-4} M, progesterone and etiocholanolone at $7{\cdot}5 \times 10^{-4}$ M. Potassium concentration of totally hemolyzed sample (distilled water) was 6·5 mEq/liter.

the first hour, release of hemoglobin by pregnanolone acetate paralleled the potassium release over the entire incubation period.

Augmentation of hemolysis by inactive compounds

Nonhemolytic steroids other than the corticosteroids were found to augment the hemolysis induced by active steroids (Table 2). In contrast to the findings in bile

TABLE 2. AUGMENTATION OF HEMOLYSIS[a]

	Epiandrosterone[c]		Allopregnanolone[c]	Androsterone[c]	Cholesterol[c]	Pregnanediol[c]	Androstenedione[c]	Cortisone[c]	Cortisol[c]	Rabbit serum 10%
Ethanol	1·3	1·4	1·0	1·3	1·3	1·5	1·5	0·7	0·5	0·6
Pregnanolone[b]	11	48	26	34	14	24	9·8	4·9	33	1·3
Pregnanedione[b]	17	59	2·5	90	11	20	36	10	39	1·2
Epipregnanolone[b]	28	94	76	86	100	83	87	59	73	1·9
Etiocholanolone	8·3	59	40	59	72	21	79	48	26	1·6
Progesterone[b]	53	95	44	100	89	52	83	100	93	1·3

[a] Numbers represent per cent hemolysis found in a typical experiment; procedure as in Table 1.
[b] Compounds added in 0·075 ml ethanol to give final concentration of $2·5 \times 10^{-4}$ M.
[c] Compounds added in 0·05 ml ethanol to give a final concentration of 5×10^{-4} M.

acid-induced hemolysis,[5] steroids with the 3-α-OH and either 5-α-H or 5-β-H configuration, including cholesterol, did not inhibit hemolysis caused by neutral steroids. These findings serve to confirm the earlier observation of Tateno and Kilbourne. Compounds with structural requirements for inhibition of bile acid-hemolysis tended, in fact, to behave like other inactive steroids, and to augment hemolysis (Table 2).

Augmentation was evident only when the hemolytic agent was present in sufficiently high concentration to be active by itself (Table 2). When the active steroid was present at hemolytic concentration, the extent of hemolysis varied directly with the concentration of augmenting steroid (Table 3). The augmentative effect of cortisol,

TABLE 3. RELATIONSHIP OF CONCENTRATION TO AUGMENTATION OF HEMOLYSIS

Compound	Concentration (M)	Hemolysis % compound alone[a]	Hemolysis %, compound + pregnanedione 5×10^{-4} M
Ethanol	(0·1 ml)	1·1	3·0
Androstenedione	1×10^{-3}	1·5	93
Androstenedione	5×10^{-4}	0·8	20
Androstenedione	1×10^{-4}	1·0	3·2
Cortisol	1×10^{-3}	1·2	70
Cortisol	5×10^{-4}	1·7	24
Cortisol	1×10^{-4}	1·4	3·1

[a] Per cent hemolysis under the conditions of Table 1.

but not of androstenedione was strongly temperature dependent; the marked augmentation present at 37° was much reduced at 24° and 4° (Table 4). Erythrocytes exposed to nonhemolytic, though augmenting, steroids lost no more potassium than controls at any steroid concentration tested (Table 5).

TABLE 4. RELATIONSHIP OF TEMPERATURE TO AUGMENTATION OF HEMOLYSIS

Compound	Concentration (M)	% Hemolysis[a]		
		37°	24°	4°
Pregnanedione	5×10^{-4}	3·0	2·2	2·1
Androstenedione	5×10^{-4}	0·8	1·1	0·8
Cortisol	5×10^{-4}	1·7	1·0	0·9
Preganedione + androstenedione	5×10^{-4}	20	33·2	2·9
Pregnanedione + cortisol	5×10^{-4}	24	3·9	3·5

[a] Extent of hemolysis after incubation at indicated temperatures. Other conditions were the same as in Table 1.

TABLE 5. RELEASE OF POTASSIUM FROM RABBIT ERYTHROCYTES

Concentration (M)	Potassium[a] (mEq/liter)		
	Ethanol control	Epiandrosterone	Cortisol
1×10^{-3}	1·3	1·3	1·4
$7{\cdot}5 \times 10^{-4}$	1·2	1·3	1·3
5×10^{-4}	1·3	1·0	1·2
$2{\cdot}5 \times 10^{-4}$	0·9	1·3	1·1

[a] Concentration of potassium, determined by flame photometer, in supernates of erythrocyte suspensions, treated as in Table 1. Complete hemolysis resulted in release of 8·75 mEq/liter.

The activity of neutral steroids was completely abolished in the presence of a 10% solution of rabbit serum. (Table 2).

DISCUSSION

The structural requirements for hemolytic activity of steroids roughly resemble those required for release of enzymes from lysosomes,[1] although the order of activity of each compound in the two systems is somewhat different. For example, testosterone is nonhemolytic but disrupts lysosomes of liver homogenates. Some of these differences may be related to differences in binding of the added steroid by protein unavoidably present in the suspensions.[9] No direct relationship was found between the solubility of steroids in the buffered saline medium and their capacity to induce hemolysis. Hemolysis, as well as other membrane activity, may in fact be a reflection of the orientation of a molecule at the lipid : aqueous interface at the surface of the cell. Such a relationship has been discussed in detail by Dingle.[10]

Evidence supporting the hypothesis that neutral steroids, at pharmacological concentrations, disrupt the membranes of cells or their organelles has been presented

in the preceding paper.[1] The actions of neutral steroids described here resemble the effects of vitamin A, which has been shown to act upon membranes in a wide variety of biological systems;[3, 10] Dingle and Lucy found that hemolysis by vitamin A was markedly concentration dependent after a critical concentration had been exceeded. Vitamin A hemolysis was maximal at 37°, diminished at 32°, was virtually abolished at 24°, and little further loss of activity was noted down to 4°. Potassium was released by vitamin A in the cold more rapidly than was hemoglobin; the hemolytic action of vitamin A was inhibited by serum.[3] These are essentially the parameters for hemolytic activity that have been established for the neutral steroids, here and elsewhere.[6, 7] Several of the steroids which disrupt lysosomes are pyrogenic in man but not in other animals, i.e. etiocholanolone, pregnanolone and pregnanedione, as well as such other 5-β-H steroids as the bile acids.[9] The 5-α-H isomers are not appreciably pyrogenic.[9] A possible relationship of membrane damage (hemolysis, rupture of lysosomes) to pyrogenicity has been discussed elsewhere.[1] There is also a specificity in hemolytic property which is determined by the nature of the end group in both types of molecule: vitamin A alcohol and aldehyde are equally active, but vitamin A acid or esters are inactive,[3] while the substitution of a hydroxyl group for a ketone at C-17 or C-21 similarly diminishes steroid activity.

Augmentation of steroid induced hemolysis by cholesterol and compounds of similar configuration (which inhibit bile acid hemolysis) may indicate that, despite their similarity in requiring the 5-β-H configuration for activity, neutral steroids and the bile acids may produce hemolysis by significantly different mechanisms. Why nonhemolytic steroids should so markedly augment the action of the hemolytic compounds remains unclear. One possibility is that these compounds block metabolic pathways which transform the active compounds into inactive ones, but the complete lack of specificity of the augmenting compounds makes this possibility unlikely. Tateno and Kilbourne[7] suggested that the augmenting corticosteroids might be "hypolytic," i.e. cause release of potassium from erythrocytes at concentrations which are too small to produce loss of hemoglobin. This possibility is highly unlikely, since red cells treated with inactive compounds, such as cortisol and epiandrosterone (steroids which markedly augment hemolysis), released no more potassium than did controls.

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