Relationship of Surfactant Properties of Some Synthetic Steroids to Bactericidal Action

By RODNEY F. SMITH, DONALD E. SHAY, and NORMAN J. DOORENBOS

Properties of seven azasteroids and a steroidal acid, which have been found to possess antibacterial activity, were compared with benzalkonium chloride (BAC). Bactericidal action against *Gaffkya tetragena*, lecithin inactivation, hemolysis of sheep erythrocytes, and the lowering of surface tension were studied. The positive relationship between surface-active properties and antibacterial activity observed with many surfactants, such as BAC, was found with only two of these steroids. Certain of the bactericidal steroids may be mechanistically similar to BAC, but the properties of the steroids studied suggest different modes of action.

SEVERAL STEROIDS have been found to possess high antimicrobial activity (1, 2). Most of these steroids were nitrogen-containing bases or their quaternary salts (quats). A recent study (3) demonstrated that the activity of these steroidal bases and quaternary salts was decreased by serum, blood, lecithin, tweens, soaps, and bile salts. Similar observations have been made on the quaternary salt antiseptics (4). The lethal action of the quaternary salt antiseptics has been attributed to their surface activity and to their ability to adsorb to negatively charged sites (5). This study was undertaken to determine whether a relationship could be established between the surface-active properties of these steroids and their antimicrobial activity. This report includes a comparison of benzalkonium chloride[1] (BAC) with eight steroids previously shown to possess antibacterial activity. The antibacterial activity of ND-15 and ND-307 has been reported (1).

EXPERIMENTAL

Test Compounds.—The structures and names of the steroids used in this study are illustrated in Fig. 1.[2]

A 100-mg. quantity of each steroid was placed into a 150-ml. bottle. Steroids 307 and 497 were dissolved by adding 5 ml. of distilled water to form a slurry. To this was added 0.1 ml. of concentrated HCl. The bottles were heated gently in a boiling water bath, with shaking, until the two compounds were completely dissolved. The total volume was then brought to 100 ml. with distilled water. Solutions of 498, 495, 436, 477, and 271 were prepared by dissolving 100-mg. samples in 5 ml. of 95% ethanol and diluting to 100 ml. with distilled water. Compound 15 was dissolved in distilled water made slightly basic with 1 *N* NaOH. A 1-mg./ml. stock solution of BAC was prepared with distilled water.

Test Culture.—The Gram-positive bacterium, *Gaffkya tetragena* ATCC 10875,[3] was used for the bactericidal experiments. The microorganism was maintained in C.T.A. medium,[4] and grown and tested in brain heart infusion broth.[4]

Antibacterial Test Conditions.—The minimal inhibitory concentrations (M.I.C.) for the steroids and BAC were determined by the conventional twofold serial dilution method. The test systems were incubated in a 37° water bath for 48 hours. The lethal studies involved the inoculation of a 24-hour culture of *G. tetragena* into 50-ml. flasks. Each flask contained broth with the test compound in a concentration of 100 mcg./ml. A second series of flasks was inoculated. They contained the test compounds in broth, to which had been added 0.05% lecithin.[5] The flasks, equilibrated at 37°, were shaken in a Dubnoff metabolic shaking water bath at 50 c.p.m. At the termination of the experiment, the flasks were transferred to an ice bath at 4°. Aliquots of the reaction broths were removed, and total plate counts were made using brain heart infusion agar.[4] Plates were incubated at 37° for 48 hours in a water-jacketed incubator and counted.

Hemolysis.—Suspensions of unhemolyzed 10% sheep erythrocytes (R.B.C.'s) were diluted with sterile saline. Test compounds and R.B.C.'s were mixed to give final concentrations of 100 mcg./ml. of each compound in 1% R.B.C.'s. The tubes and controls were incubated at 37° and observed for hemolysis for up to 16 hours.

Surface Tension Measurements.—Surface tension measurements of the test compounds were determined with the DuNouy tensimeter. All measurements were made at 25° with triplicate readings.

RESULTS

The minimal inhibitory concentration measurements (M.I.C.) shown in Table I revealed that the five aza-cholestanes, 307, 497, 498, 495, and 271, were active. The aza-pregnane derivative 477 was active also, while the aza-androstenol compound 436 and the keto acid 15 were not inhibitory.

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[1] Marketed as Zephiran chloride by Winthrop Laboratories, Inc., New York, N. Y.

[2] The steroids represent eight of a group prepared by Norman J. Doorenbos, Department of Pharmaceutical Chemistry, School of Pharmacy, University of Maryland. In previous publications the steroid code numbers have been prefixed with ND.

[3] American Type Culture Collection, Washington, D. C.

[4] Baltimore Biological Laboratories, Inc., Baltimore, Md.

[5] Nutritional Biochemical Corp., Cleveland, Ohio.

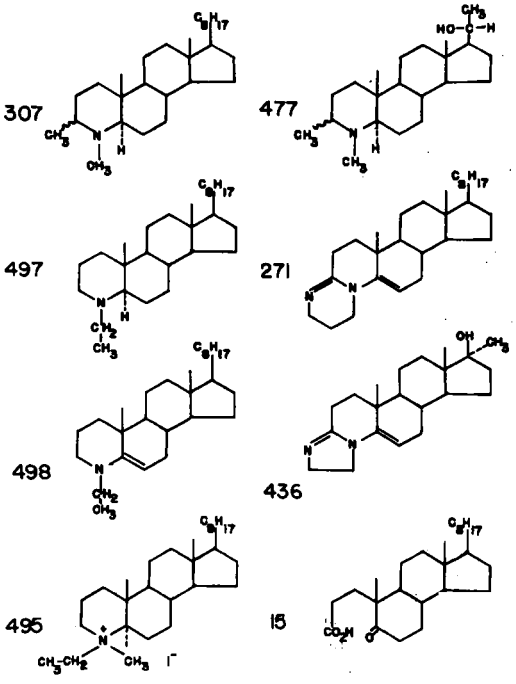

Fig. 1.—Structures of the eight steroids evaluated.

307, 3xi,4-Dimethyl-4-aza-5α-cholestane (6).
497, 4-Ethyl-4-aza-5-α-cholestane (6).
498, 4-Ethyl-4-aza-5-cholestene (6).
495, 4-Ethyl-4-aza-5α-cholestane methiodide (6).
477, 3xi,4-Dimethyl-4-aza-5α-pregnan-20β-ol (6).
271, 1′,4′,5′,6′,-Tetrahydropyrimidino[a-4,3]-4-aza-5-cholestene (7).
436, 2′-Imidazolino[a-4,3]-17α-methyl-4-4-aza-5-androsten-17β-ol (7).
15, 3,5-Seco-4-nor-5-cholestanon-3-oic acid (8).

The lethal action of the steroids and BAC is shown in Table II. Generally, there was agreement between the M.I.C. values (Table I) and the relative killing action of the active steroids.

The reversal effects of the lecithin (0.05%) on the lethal action of the steroids suggest the formation of lecithin-steroid complexes. BAC, which is known to be inactivated by phospholipids (5), presumably was used in a concentration too high to be totally inactivated by 0.05% lecithin. The appearance of turbidity in the lecithin flasks, which were incubated for 48 hours, revealed that 307, 477, and 271 were completely inactivated. The absence of turbidity in the flasks containing 497, 498, and 495 showed that these compounds were only partially inactivated, demonstrating that a substantial concentration of free steroid inhibited or killed the remaining viable cell population.

The relationship of lethal action to other properties regarded as important in the action of the quats is shown in Table III. None of the active steroids caused complete disruption of sheep R.B.C.'s. The 2+ reaction demonstrated marked extrusion of hemoglobin; but approximately 50% of the cells were still intact after 16 hours. This was observed by measuring the volume of packed cells that settled out of suspension. The weakest of the lethal steroids, 477, produced only a trace of hemolysis. The compound 436 was not hemolytic, while 15 caused complete hemolysis spontaneously, as was previously reported (3). Neither 436 nor 15 was inhibitory or lethal to the organism.

It should be noted that all surface tension values for steroids were higher in broth than in distilled water. Perhaps materials in the broth removed portions of the steroids from solution by complex formation. The results given in Table III indicate no consistency among lethal action, hemolysis, and lowering of surface tension by the steroids.

A summary of the results obtained in this study can be seen in Table IV. BAC, 307, and 497, representing Type 1, are positive in all categories studied. Compounds 498, 495, and 271 (Type 2) resemble the BAC group but deviate in surface

Table I.—Minimal Concentration of Test Compounds Inhibitory to *G. tetragena*

Test Compd.		mcg./ml.
Steroids	307	0.0185
	497	3.10
	498	0.185
	495	0.185
	271	0.75
	477	25.00
	436	...
	15	...
BAC		0.01

Table II.—Comparative Bactericidal Action of Test Compounds with Growing Cells of *G. tetragena*[a]

Test Compd.		Viable Cell Count/ml. Plain Broth	Viable Cell Count/ml. With 0.05% Lecithin	Growth After 48 Hr.[c]
Steroids[b]	307	600	246,000	+
	497	100	101,000	−
	498	10	115,000	−
	495	10	186,000	−
	477	10,000	575,000	+
	271	100	920,000	+
BAC		10	100	−

[a] Cells were exposed to compounds (100 mcg./ml.) for 3 hours at 37° C. Initial viable cell concentration was 810,000/ml. [b] 436 and 15 had no inhibitory or lethal effects. [c] Lecithin flasks were incubated 48 hours to observe for recovery of viable cells.

Table III.—Comparison of Lethal Steroid Action to Hemolytic and Surface Tension Reduction

Test Compd. (100 mcg./ml.)		Lethal Action	Hemolysis[a] 1 Hr.	Hemolysis[a] 16 Hrs.	Surface Tension (dynes/cm.)[b] Distilled Water	Surface Tension (dynes/cm.)[b] Broth
Steroids	307	+	2+	2+	39	44
	497	+	1+	2+	38	44
	498	+	2+	2+	51	52
	495	+	2+	2+	44	46
	477	+	0	1	58	61
	271	+	2+	2+	44	61
	436	−	0	0	62	70
	15	−	4+	4+	37	42
BAC		+	2+	2+	38	38

[a] Results are given as relative values compared to control systems. Hemolysis: 4+, complete; 3+ and 2+, partial; 1+, trace; 0, negative: no hemolysis detected. [b] Distilled water control, 70 dynes/cm.; broth medium control, 60 dynes/cm.

TABLE IV.—SUMMARY OF EXPERIMENTAL DATA

Type	Compd.	Lethal Action	Hemolysis	Surface Action	Lecithin Inactivation
1	BAC	+	+	+	±[a]
	307	+	+	+	+
	497	+	+	+	+
2	498	+	+	±	+
	495	+	+	±	+
	271	+	+	±	+
3	15	−	+	+	−
4	477	+	−	−	+
5	436	−	−	−	−

[a] ±, Weakly positive.

action. Further dissimilarities to BAC can be noted in the categories of lethal action and lecithin inactivation, as exhibited by 15 (Type 3), and in hemolytic and surface action, demonstrated by 477 (Type 4). In none of the categories does 436 (Type 5) compare with the qualities of BAC.

DISCUSSION

Both naturally occurring and synthetic steroids have been known to exert inhibitory effects on microorganisms. Arnaudi (9) suggested that lowering of surface tension by cholesterol might induce antibacterial action. It is generally assumed that the inhibitory effects of fatty acids are due to their surface activity, which causes nonspecific alterations in cell permeability (10). The relationship of surface action and permeability damage to the lethal action of the quats has been recognized by Hotchkiss (11).

Hemolytic action on erythrocytes is considered to be indicative of permeability damage (12), and lowering of surface tension usually suggests the probability that hemolysis will occur (13). The complex physiochemical changes that surround membrane alterations have been studied with many detergent substances.

The steroids described in this report exhibited one or more properties characteristic of most surfactant-type compounds, suggesting that they may be mechanistically related to lethal compounds that act by altering membranes.

Partial or complete nullification of lethal action by lecithin is indicative of cationic quats (5) and is shared by all of the lethal steroids studied here. Evidence points to phospholipids as the membranous binding sites for quats (14). The inactivation of lethal agents by exogenous substances may be looked upon as a complex formation which mimics the true cell receptor sites for the lethal agent. This concept has held true for nystatin-ergosterol interactions (15, 16). Other systems of lethal agents, such as the steroids studied here and an exogenous inactivator (lecithin), may be quite nonspecific and misleading.

While some surface-membrane action might appear to be the most obvious mechanism, each steroid-bacterial cell union is highly specific. Not all of the steroids active against *G. tetragena* will inhibit or destroy other related Gram-positive bacteria (17). Moreover, some quaternary ammonium steroids have been completely inactive against bacteria in screening tests (18).

More detailed studies on steroids will be required to delineate a physiochemical-biochemical mechanism of action.

SUMMARY

Eight synthetic steroids were compared with BAC for their bactericidal action against *G. tetragena*. The hemolysis of sheep erythrocytes, lowering of surface tension, and inactivation by lecithin were studied. The properties of two aza-cholestanes were similar to those of BAC. The remaining steroids were classified into four types, depending upon their properties. A positive relationship between antimicrobial activity and surface or hemolytic activities was not found. Since the lethal action of the quats is attributed to these properties, it is doubtful that these steroids and quats act by an identical mechanism. Nevertheless, since these steroids are surface-active agents, their lethal effects may be assisted by this property.

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