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PHYSIOLOGICAL ACTIVITY OF WATER BEETLE DEFENSIVE AGENTS. I. TOXICITY AND ANESTHETIC ACTIVITY OF STEROIDS AND NORSESQUITERPENES ADMINISTERED IN SOLUTION TO THE MINNOW *Pimephales promelas* Raf.

JAMES R. MILLER and RALPH O. MUMMA

Department of Entomology
and the Pesticide Research Laboratory and Graduate Study Center
The Pennsylvania State University, University Park, Pennsylvania 16802

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Abstract—By means of a bioassay employing the minnow *Pimephales promelas*, the anesthetic activities and toxicities of various defensive steroids and norsesquiterpenes produced by the Dytiscidae and Gyrinidae were compared with those of a wide selection of steroid standards. The most widely occurring major components of dytiscid secretions, 4-pregnen-3-ones and related derivatives, were among those compounds most active in minnow bioassays. The norsesquiterpenes gyrinidal and gyrinidione were among the most toxic compounds tested but they possessed little anesthetic action. The anesthetic activity of gyrinidone was comparable to that of testosterone. Steroid activity in the minnow bioassay was highly related to the degree of oxygenation; steroids oxygenated only at the termini of the molecule were most active. Less or additional oxygenation resulted in a loss of activity. When steroids were rapidly administered to minnows the activities of many of them were similar, suggesting they share a common mode of action.

Key Words—Dytiscidae, Gyrinidae, chemical defense, 4-pregnen-3-ones, gyrinidal, gyrinidione, gyrinidone, steroids, norsesquiterpenes.

INTRODUCTION

Aquatic beetles of the families Dytiscidae and Gyrinidae possess defensive glands which secrete agents repellent and toxic to vertebrate predators, i.e., fishes and amphibians. The prothoracic defensive secretions of numerous dytiscid species primarily contain steroids (Table 1, A), many of which are

TABLE 1. DEFENSIVE AGENTS OF AQUATIC COLEOPTERA
A. Dytiscid prothoracic defensive compounds

Compound	Species	Quantity (μg/beetle)[a]	References
4-pregnen-21-o1-3,20-dione (deoxy-corticosterone, DOC)	*Acilius semisulcatus*	(M)	Miller and Mumma (unpublished)
	Acilius sulcatus	19 (m)	Schildknecht et al. (1967b) Schildknecht (1970)
	Agabus bipustulatus	(M)	Schildknecht and Hotz (1970)
	Agabus seriatus	40 (M)	Miller and Mumma (1973)
	Cybister confusus	(m)	Chadha et al. (1970)
	Cybister limbatus	133 (M)	Sipahimalani et al. (1970)
	Cybister tripunctatus	143 (M)	Chadha et al. (1970)
	Dytiscus marginalis	400 (M)	Schildknecht et al. (1966)
	Graphoderus liberus	20 (M)	Miller and Mumma (1973)
4-pregnen-20α-o1-3-one	*Acilius sulcatus*	1 (m)	Schildknecht et al. (1967b) Schildknecht (1970)
	Cybister limbatus	8 (m)	Sipahimalani et al. (1970)
	Dytiscus marginalis	(m)	Schildknecht and Hotz (1967)
4-pregnen-20β-o1-3-one	*Cybister tripunctatus*	100 (M)	Chadha et al. (1970)
	Ilybius fenestratus	1 (t)	Schildknecht and Birringer (1969)
4,6-pregnadien-21-o1-3,20-dione	*Acilius sulcatus*	56 (M)	Schildknecht et al. (1967b) Schildknecht (1970)
	Cybister literalimarginalis	(m)	Schildknecht (1968)
	Cybister limbatus	13 (m)	Sipahimalani et al. (1970)
	Cybister sp.	(t)	Schildknecht and Körnig (1968)
4,6-pregnadien-20α-o1-3-one (cybisterone)	*Acilius sulcatus*	7 (m)	Schildknecht et al. (1967b) Schildknecht (1970)
	Cybister lateralimarginalis	167 (M)	Schildknecht et al. (1967c)

	Cybister limbatus	17 (m)	Sipahimalani et al. (1970)
	Dytiscus marginalis	(t)	Schildknecht and Hotz (1967)
4,6-pregnadien-3,20-dione	*Acilius sulcatus*	6 (m)	Schildknecht et al. (1967b)
			Schildknecht (1970)
	Cybister lateralimarginalis		Schildknecht (1968)
4,6-pregnadien-12β,20-diol-3-one	*Cybister lateralimarginalis*	(m)	Schildknecht (1968)
4-pregnen-12β-o1-3,20-dione	*Cybister limbatus*	80 (m)	Chadha et al. (1970)
4,6-pregnadien-12β-o1-3,20-dione	*Cybister limbatus*	37 (M)	Chadha et al. (1970)
(cybisterol)	*Cybister* sp.	1000 (M)	Schildknecht and Körnig (1968)
4-pregnen-15α,20β-diol-3-one	*Platambus maculatus*	10 (M)	Schildknecht et al. (1969b)
4,6-pregnadien-3,20-dione-15α isobutyrate	*Agabus sturmi*		Schildknecht and Hotz (1971)
4,6-pregnadien-15α-o1-3-one-20β-isobutyrate	*Agabus sturmi*		Schildknecht and Hotz (1971)
4-pregnen-15α-o1-3,20-dione-7α isobutyrate	*Agabus sturmi*		Schildknecht and Hotz (1971)
4-pregnen-15α-o1-3,20-dione-7α hydroxyisobutyrate	*Agabus sturmi*		Schildknecht and Hotz (1971)
4-androsten-17β-o1-3-one	*Ilybius fenestratus*	28 (m)	Schildknecht and Birringer (1969)
(testosterone)	*Ilybius fuliginosus*		Schildknecht et al. (1967a)
1,4-androstadien-17β-o1-3-one (1-dehydrotestosterone)	*Ilybius fenestratus*	16 (m)	Schildknecht and Birringer (1969)
1,3,5-androstatrien-3β,17β-diol (estradiol)	*Ilybius fenestratus*	19 (m)	Schildknecht and Birringer (1969)
1,3,5-androstatrien-3β-o1-17-one (estrone)	*Ilybius fenestratus*	2 (t)	Schildknecht and Birringer (1969)

B. Gyrinid pygidial defensive compounds

Compound	Species	Quantity (μg/beetle)[a]	References
gyrinidial	*Dineutus assimilis*	118 (M)	Miller et al. (1975)
	Dineutus hornii		Meinwald et al. (1972)
	Dineutus nigrior	66 (M)	Miller et al. (1975)
	Dineutus serrulatus	ca. 100 (M)	Meinwald et al. (1972)
	Gyrinus marinus		Schildknecht et al. (1972)
	Gyrinus minutus		Schildknecht et al. (1972)
	Gyrinus natator	ca. 84 (M)	Schildknecht et al. (1972)
	Gyrinus substriatus		Schildknecht et al. (1972)
	Gyrinus ventralis	80 (M)	Meinwald et al. (1972)
gyrinidione	*Dineutus assimilis*	95 (M)	Miller et al. (1975)
	Dineutus nigrior	54 (M)	Miller et al. (1975)
gyrinidone	*Dineutus assimilis*	22 (m)	Miller et al. (1975)
	Dineutus discolor	(M)	Wheeler et al. (1972)
	Dineutus nigrior	12 (m)	Miller et al. (1975)

[a] μg per beetle are presented when reported. The relative concentration per beetle is as follows: M = major, m = minor, and t = trace.

stored in surprisingly high quantities. Although dytiscid secretions may contain a wide variety of steroid structures, including such well-known mammalian hormones as deoxycorticosterone (DOC), testosterone, and estradiol, the compounds in greatest quantity are generally 4-pregnen-3-ones, 4,6-pregnadien-3-ones, or the many modifications of these basic structures. Several nonsteroidal compounds have been isolated as major components of prothoracic defensive secretions of several dytiscid species of the subfamily Colymbetinae (Schildknecht et al., 1969a; Schildknecht and Tacheci, 1971). The pygidial secretions of the Gyrinidae are composed largely of oxygenated norsesquiterpenes (Table 1, B), which have been shown by Benfield (1972) to be repellent to fishes and amphibians and by Miller et al. (1975) to be toxic as well.

The discovery that aquatic beetles utilize steroids and norsesquiterpenes as defensive agents raises the question of how these compounds function in a defensive context. In addition to their classical hormonal actions, various pharmacological effects of steroids on animals are recognized, such as steroid anesthesia, allergy, fever, and immunological reactivity as well as steroid hematopoietic, hemolytic, and cytotoxic properties (Kappas and Palmer, 1963).

Although it is not yet certain which toxicological action(s) of steroids is being exploited by the beetles in their defense against predation, observations (Schildknecht et al., 1966; Schildknecht et al., 1967a; 1967b; Miller et al., 1975) indicate that anesthesia may be a primary effect of the agents elaborated by water beetle defensive glands. Fish and frogs, force-fed beetle defensive secretions, show signs of excitation followed by varying degrees of narcosis, which is usually reversible. Likewise, when fish are placed in solutions of defensive steroids at concentrations of 5–10 μg/ml they enter a state of deep narcosis in ca. 30 min. The effects may be completely reversed if fish are then placed in fresh water. Fish that remain in such steroid solutions are usually killed (Miller et al., 1975).

As a means of screening steroids for their comparative anesthetic potency, Selye and Heard (1943) exposed the redfin shiner, *Notropis cornutus*, to solutions of various steroids and recorded the minimal dosage required to cause minnows to lose their ability to orient in water currents. These experiments proved that just as various neutral steroids are anesthetic to mammals (Selye, 1941a; Selye, 1941b; Selye, 1942) they are also capable of anesthetizing fish.

The present study was undertaken in order to expand the available information on the physiological action of steroids on fish. A minnow bioassay similar to that of Selye and Heard (1943) was used to measure both the relative anesthetic activity and relative toxicity of a wide selection of steroids to the minnow, *Pimephales promelas*. Various types of steroids similar to those produced by dytiscids were tested as were the gyrinid norsesquiterpenes. The

activities of these compounds were measured at concentrations far in excess of the AC_{50} (anesthetic concentration) and LC_{50} (lethal concentration) values by recording lag times for anesthesia and death. It was hoped that a measure of commonness in the mode of action of steroids could be obtained by quantifying their activities when administered at rates where varying detoxification mechanisms and rates would be inconsequential. The data obtained from the study taken together with those of Selye and Heard (1943) will hopefully serve to further characterize the physiological activity of steroids and norsesquiterpenes in their role as defensive agents.

METHODS AND MATERIALS

Experimental Animals

The fathead minnow, *Pimephales promelas* Raf., was used in all experiments because of its desirable small size and commercial availability. Minnows of a mean body length of 4.5 cm and a mean weight of 1.2 g (range 0.7–1.7 g) were purchased from live-bait dealers in the vicinity of State College, Pennsylvania. In order to standardize them, all batches of minnows were stored at 4°C in 40-gal aquaria for at least 48 h before they were used in experiments. During the period of storage, minnows were constantly aerated but were not fed. Experiments conducted on such minnows upon their acclimatization to near room temperatures yielded uniform results. Once minnows were used in an experiment they were discarded, since it had been shown (Kappas and Palmer, 1963) that adaptation to the anesthetic effect of steroids could occur in animals repeatedly treated with these substances.

Test Compounds

Steroids of a purity >99% were purchased from various commercial sources. Their purity was checked by thin-layer chromatography employing multiple development.

Norsesquiterpenes were isolated from the gyrinids, *Dineutus assimilis* and *Dineutus nigrior* (Miller et al., 1975). Purities of norsesquiterpenes used in this study ranged from 97% to 99% as ascertained by gas–liquid chromatography.

Experimental Procedure

In order to determine the physiological activities of the test compounds both at threshold levels and above, minnows were exposed to a range of toxicant concentrations from ca. 1 to 100 μg/ml. The lag time from administra-

tion of the compounds until minnows lost their equilibrium and could no longer right themselves was taken to be a measure of anesthetic activity. The lag time until respiratory arrest (RA) or cessation of gill-pumping was taken to be a measure of toxicity. Although in some cases heartbeat continued for at least 15 min after RA, cessation of gill-pumping was selected as the end-point of the toxicity bioassay because it was far more readily observed. Curves of lag times vs. toxicant concentration were obtained along with a concentration range bounding the AC_{50} and LC_{50} values.

In the experimental procedure ca. 80 minnows at a time were allowed to equilibrate from 4°C to 25±0.5°C over the period of 1 h and were held at 25°C for another 2 h. Three minnows were then transferred to each of a series of 250-ml glass beakers containing 50 ml of aerated tap water held at 25± 0.5°C by means of a water bath. From stock solutions of the steroids and norsesquiterpenes prepared in ethanol or DMF (67 μg/μl solvent), the appropriate quantities of toxicant needed to produce the desired range of concentrations were injected into the beakers containing fish and the solutions were stirred thoroughly. Control experiments demonstrated no measurable difference in the toxicity of solutions of these compounds when they were prepared using the carrier ethanol, the carrier DMF, or when the solution was prepared without carrier by exhaustively shaking crystalline compounds in water.

Using a completely randomized experimental design, 40 compounds were tested on *P. promelas* in the above manner. Each concentration was replicated at least three times. Additionally, the constancy of response of each batch of minnows acclimated to 25°C was monitored by measuring their survival time in a standard solution of the steroid DOC at 10 μg/ml. The mean survival time of all batches of minnows exposed to 10 μg/ml DOC was 27±3.1 (SD) min.

RESULTS AND DISCUSSION

Data typical of those obtained in this study are presented graphically in Fig. 1 as a log–log plot of lag times for anesthesia and RA vs. toxicant concentration. Such a double logarithmic plot yields a straight line over the higher range of toxicant concentrations, and a straight line in this case is characteristic of an absorption process, following the Freundlich absorption isotherm (Rothblat et al., 1966). As the AC_{50} or LC_{50} values were approached, lag times progressively diverged from the Freundlich isotherm, presumably because at these concentrations detoxification mechanisms significantly influenced the effects of the substances tested. The effects of all compounds administered to *P. promelas* were short term. Death rarely occurred after the

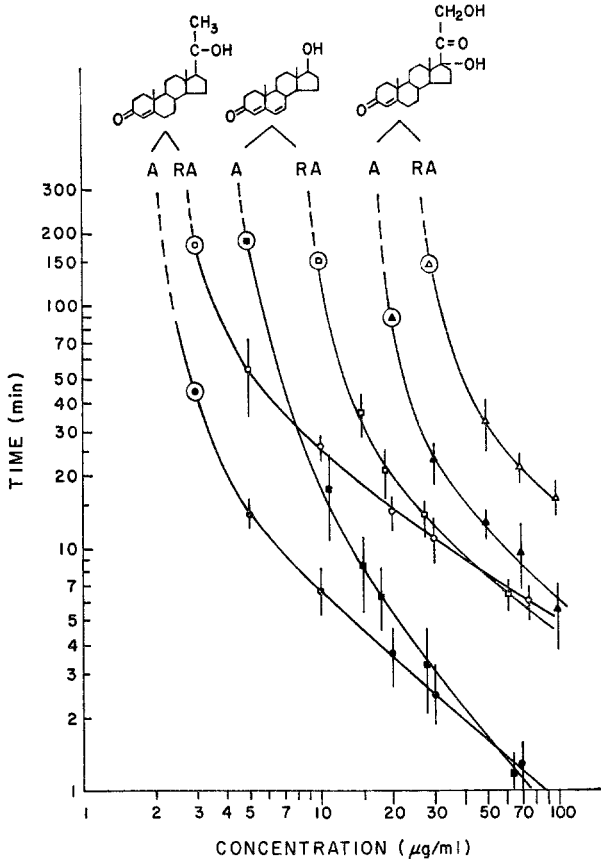

FIG. 1. Dose–response curves typical of highly active, active and slightly active steroids. Highly active = 4-pregnen-20β-ol-3-one (2), active = 4,6-androstadien-17β-ol-3-one (13), and slightly active = 4-pregnen-17α, 21-diol-3,20-dione (17). A = anesthesia and RA = respiratory arrest. Circled means were calculated upon exclusion of some (<50%) unanesthetized or surviving minnows.

5-h period over which data were recorded for these experiments. In fact, minnows anesthetized but not killed usually had made or were making a recovery by 5 h.

Although the activities of the test compounds were measured at seven or eight different concentrations, the findings are adequately summarized in the abbreviated Table 2. Compounds are arranged according to increasing toxicity and are arbitrarily grouped into these categories: highly active, active,

slightly active, and inactive. The data for the gyrinid norsesquiterpenes are presented separately at the end of Table 2.

AC_{50} values were found to parallel LC_{50} values, and in both cases the following generalizations can be made concerning structure–activity relationships: The activity of compounds tested by the *P. promelas* bioassay was highly dependent on their water solubility. Compounds roughly fell into three categories: those very water insoluble (< 5 μg/ml) and inactive, those slightly soluble in water (ca. 10–100 μg/ml) and highly active or active, and those fairly soluble in water (> 200 μg/ml) and inactive or slightly active.

Several types of compounds fell into the very insoluble group; included are those compounds oxygenated at only one terminus of the molecule (C-3 and C-17 or -20) whether androstanes (31,32) or pregnanes (39), the androstan- and androsten-3,17-diols (30,33), most of the estratrienes (36, 37, 38), and the singly oxygenated cholestenes (34, 35). Since the uptake of steroids into the fish is concentration dependent (see Fig. 1), the rate of uptake for these compounds, even from a saturated solution, may have been too slow to effect any accumulation of these materials within the fish. Therefore, no judgement can be passed as to the potential activity of highly water-insoluble compounds. Judging from the comparative studies of the anesthetic activity of steroids injected intraperitoneally into rats (Selye, 1942), some of these steroids are weakly anesthetic.

The compounds generally anesthetic and toxic to *P. promelas* were oxygenated at the termini (C-3, C-17, C-20, or C-21) of the molecule. The active androstanes were oxygenated at C-3 and C-17, while the active pregnanes were oxygenated at C-3 and C-20 or C-21. The highly active steroids of those tested in this study were terminally oxygenated pregnenes and 5α(*cis*)-androstanes. Although not included here, terminally oxygenated 5α and β pregnanes are even more active in mammals than corresponding pregnenes (Selye, 1942; Selye et al., 1943; Figdor et al., 1957). Terminally oxygenated androstenes were consistently less active than the pregnenes and androstanes (see compounds 10–15).

The degree of ring unsaturation beyond the $\Delta 4$ unsaturation, as just discussed, influenced activity only slightly. The activities of testosterone (12), 1-dehydrotestosterone (15), and 6-dehydrotestosterone (13) were very similar. Unsaturation of progesterone (1) in the D ring at C-16 did result in a loss of activity (7).

The steric positions of hydroxyl groups were also of only slight importance as testosterone (12) and epitestosterone (11) possessed similar activity. The activities of (2) and its α isomer (tested only at a few selected concentrations, and these data are not included in Table 2) were also very similar.

Oxygenation of steroids in positions in addition to the termini can greatly reduce activity; however, some positions on the rings are much more critical

TABLE 2. ANESTHETIC ACTIVITY AND TOXICITY OF SELECTED STEROIDS AND NORSESQUITERPENES AS MEASURED BY A MINNOW BIOASSAY

Code No.	Compound	Lag time (min)[a] 70 μg/ml A	70 μg/ml RA	20 μg/ml A	20 μg/ml RA	10 μg/ml A	10 μg/ml RA	AC_{50} (μg/ml)	LC_{50} (μg/ml)
	Highly active steroids								
(1)	4-pregnen-3,20-dione (progesterone)	1.0	6.0	2.6	15.1	5.9	22.6	1–2	1–2
(2)	4-pregnen-20β-ol-3-one	1.3	8.0	3.7	13.4	6.8	26.8	1–3	1–3
(3)	5α-androstan-3α-ol-17-one (androsterone)	1.0	5.7	1.0	9.7	1.5	13.2	1–2	2–3
(4)	4-pregnen-21-ol-3,20-dione (deoxycorticosterone)	1.1	6.7	2.6	14.2	5.8	28.0	1–3	2–3
(5)	3,4-di-*p*-hydroxyphenylhex-3-ene (diethylstilbestrol)	1.3[b]	5.7[b]	2.0[b]	8.4[b]	2.9	21.1	1–3	3–5
(6)	5α-androstan-3,17-dione (androstanedione)	1.1	7.1	2.4	12.7	6.5	23.9	1–3	3–5
(7)	4,16-pregnadien-3,20-dione (16-dehydroprogesterone)	1.1	6.9	2.8	14.1	5.5	32.7	3–5	3–5
	Active steroids								
(8)	5β-pregnan-12α-ol-3,20-dione	2.2	9.4	6.9	25.8	17.0	39.1	3–5	5–10
(9)	1,3,5(10)-estratrien-3,17α-diol (estradiol)	2.4[b]	17.9[b]	4.8[b]	27.4	16.2	58.5	5–10	5–10
(10)	4-androsten-3,17-dione (androstenedione)	1.1	6.7	3.5	21.6	14.5	99.6	3–5	5–10
(11)	4-androsten-17α-ol-3-one (epitestosterone)	1.2[b]	8.3[b]	4.2	18.7	17.0	126.2	3–5	5–10
(12)	4-androsten-17β-ol-3-one (testosterone)	1.3	6.5	4.4	16.2	16.4	s	3–5	5–10
(13)	4,6-androstadien-17β-ol-3-one (6-dehydrotestosterone)	1.2	6.3	6.0	17.9	22.0	s	3–5	5–10
(14)	5-androsten-3β-ol-17-one (dehydroisoandrosterone)	2.0	7.0	6.3	18.6	23.1	s	5–10	5–10
(15)	1,4-androstadien-17β-ol-3-one (1-dehydrotestosterone)	1.1	6.9	6.3	18.8	23.3	s	3–5	5–10
(16)	4-pregnen-3,11,20-trione (11-ketoprogesterone)	1.4	9.8	11.3	35.6	65.3	s	5–10	5–10

Slightly active steroids								
(17) 4-pregnen-17α,21-diol-3,20-dione (deoxycortisol)	9.4	21.0	u	S	U	S	10–20	20–30
(18) 4-pregnen-21-ol-3,11,20-trione (11-dehydrocorticosterone)	60.4	129.0	U	S	U	S	30–50	30–50
(19) 4-pregnen-11β,21-diol-3,20-dione (corticosterone)	49.3	83.5	U	S	U	S	30–50	30–50
(20) 4-androsten-3,11,17-trione (androstenetrione)	11.5	116.4	U	S	U	S	30–50	50–70
(21) 4-androsten-17β-ol-3,11-dione (11-ketotestosterone)	43.0	s	u	S	U	S	30–50	50–70
Inactive steroids[c]								
Norsesquiterpenes								
(27) gyrinidione	6.1	10.1	16.2	24.0	25.2	34.9	1–3	1–3
(28) gyrinidal	8.3	11.5	20.4	26.4	31.2	38.2	1–3	1–3
(29) gyrinidone	2.5	12.5	4.0	45.3	8.9	s	5–10	10–15

[a] A = anesthesia; RA = respiratory arrest; s = < 50% survived; S = > 50% survived; u = < 50% not anesthetized; U = > 50% not anesthetized.

[b] Concentration exceeds the solubility limit.

[c] Water soluble and inactive: 5β-cholanic acid-3α,7α,12α-triol (cholic acid) (22); 4-pregnen-18-ol-11β-21-diol-3,20-dione (aldosterone) (23); 4-pregnen-3,20-dione-21-sulfate (24); 4-pregnen-17α,21-diol-3,11,20-trione (cortisone) (25); 4-pregnen-11β,17α,21-triol-3,20-dione (cortisol) (26). Water insoluble and inactive: 5α-androstan-3β,17β-diol (30); 5α-androstan-3β-ol (31); 5α-androstan-17-one (32); 5α-androsten-3β,17β-diol (33); 5-cholesten-3β-ol (34); 4-cholesten-3-one (35); 1,3,5(10)-estratrien-17α-ol-3-benzoate (36); 1,3,5(10)-estratrien-3,16α,17β-triol (37); 1,3,5(10)-estratrien-3β-ol-17-one (38); 5α-pregnan-3β-ol(39); 5-pregnen-3-ol-20-one (40).

than others. The effect of oxygenations at the C-11 β position in reducing activity can be appreciated by comparing these compound pairs: (1) and (16), (4) and (18), (4) and (19), (10) and (20), and (12) and (21). Addition of oxygen to the C-17 resulted in a lesser reduction in activity (compare 4 and 17). Compound (8) contains a C-12 α-OH but yet is quite active. The parent 5β-pregnan-3,20-dione could be expected to be slightly more active than progesterone (1) (Figdor et al., 1957). Likewise, addition of OH at C-6 of progesterone and DOC acetate caused only a moderate reduction in activity in rat tests (Selye, 1942).

Soluble but inactive compounds were multiple oxygenated. It has already been pointed out that the addition of 17 α-OH, 11-keto, or 11-OH to DOC (4) greatly reduced activity. As seen by compounds 25 and 26, addition of the combination of 11-keto or OH plus a 17 α-OH resulted in a total loss of activity. Aldosterone (23) and cholic acid (22) are multiple oxygenated and inactive. The addition of the very polar sulfate group to the C-21 hydroxyl of DOC (4) completely inhibited activity (24).

Despite the fact that norsesquiterpenes (Table 1, B) are structurally dissimilar to steroids, their toxicities as measured by *P. promelas* were comparable. The straight chain gyrinidal and the monocyclic gyrinidione, each possessing an aldehyde and two keto moieties, fall into the highly active group of compounds. The bicyclic gyrinidone, possessing a keto and hydroxyl group in a hemiacetal linkage and one additional keto group, falls into the active group.

Like the steroids, gyrinidone was capable of inducing a prolonged and very deep anesthesia that was completely reversible. Judging from the ratio of AC_{50} to LC_{50}, the therapeutic index for gyrinidone compares favorably with that of many of the steroids (compare 29 with 12–16). When the ratios of lag times for respiratory arrest to lag times for anesthesia are compared at a given concentration such as 70 μg/ml it can be seen that values for steroids are about 5–6 and that of gyrinidone is also about 5.

Although gyrinidal and gyrinidione were highly toxic to *P. promelas*, the anesthetic qualities of these compounds were less evident than those for gyrinidone or the steroids. When gyrinidal and gyrinidione caused minnows to lose their equilibrium, minnows never became totally quiescent and RA occurred very soon after equilibrium loss. Ratios of lag times for RA to lag times for anesthesia at 70 μg/ml are much lower for gyrinidal and gyrinidione than gyrinidone (1.4 and 1.6 vs. 5). Once equilibrium loss had occurred in minnows exposed to gyrinidal and gyrinidione, recovery was poor even after these minnows were then transferred to fresh water. On various occasions gyrinidal and gyrinidione treated minnows bled from the gills at about the time death occurred. From our observations these two compounds are more toxic in nature than anesthetic.

The lag times for steroid anesthesia and RA presented in Table 2 provide a means for comparing the activities of compounds when they are administered at increasing concentrations. As toxicant concentration increased, a convergence of lag times of the highly active and active steroids occurred [see Fig. 1 where compound (2) is representative of the highly active group and compound (13) of the active group]. At 70 μg/ml the lag times for a great number of terminally oxygenated steroids were nearly identical. Lag times of multiple oxygenated compounds such as (17) did not converge with those of the active compounds.

It can be suggested, then, that provided deactivation mechanisms are not a factor, either the absorption rates and activities of active compounds are quite similar or else differences in absorption rates happened to be offset by compensating differences in activity. We favor the former explanation and its implication that the activity of steroids is a very nonspecific phenomenon where similar quantities of many steroids elicit the same generalized effect.

Even though their AC_{50} and LC_{50} values were similar, lag times for a few steroids in the highly active group were distinctly different from the others. Most notable is androsterone (3) for which at 10 μg/ml lag times were significantly shorter than for compounds (1), (2), (4), (6), and (7) (P0.01). Figdor et al. (1957) reported similar findings in the mouse, where the onset of anesthesia for some steroids varied quite widely from others. It was proposed that varying times for the onset of anesthesia were due to differences in rates of steroid uptake brought about by differences in molecular configuration and solubilities; however, no clear-cut relationship was outlined. Likewise, compounds 3 and 5 in Table 2 may have been absorbed more rapidly than the others by *P. promelas*.

Reexamination of Table 1 in light of the data presented in Table 2 leads one to conclude that the defensive arsenal of dytiscids has been so selected to include only those steroids highly anesthetic and toxic to fish. The compounds most widely occurring as major components of dytiscid secretions, 4-pregnen-3-ones and 4,6-pregnandien-3-ones, were among those compounds most active in minnow bioassays. Although steroids oxygenated in positions in addition to the termini are produced as major components of the defensive secretions of some dytiscid species, such oxygenation never occurs at C-11 or C-17 in the pregnenes or pregnadienes. Interestingly, the C-7, -12, and -15 positions are additionally hydroxylated, and in some cases such hydroxyl groups occur as isobutyrate derivatives.

Preliminary bioassays conducted by Schildknecht and co-workers indicate that the biological activities of dytiscid 4-pregnenes, 4,6-pregnadienes, and their ring hydroxylated derivatives are very similar (Schildknecht et al., 1967b; Schildknecht and Hotz, 1971). When administered in solution to goldfish these compounds have little or no effect at 1 μg/ml. At 2 μg/ml

goldfish are slightly narcotized, and at 10 μg/ml they are deeply anesthetized.

It is rare that 4-androstenes are found in dytiscid secretions, and they are never the major components. As previously suggested in this paper, their apparent higher rate of detoxification may make them less efficient toxins. Estradiol and estrone were the only highly water-insoluble steroids found in dytiscid secretions. In the one beetle in which they were found, *Ilybius fenestratus*, they occurred only in minor or trace amounts.

CONCLUSIONS

Under natural conditions water beetles presumably administer their defensive agents to a predator via the digestive track (Blunck, 1917). In the present study, test compounds were administered in external solution to fish, and the results may therefore not be directly applicable to the natural situation. However, on the basis of the data that have now been accumulated, it can be suggested that the Dytiscidae are exploiting the anesthetic action of steroids in their defense. Dytiscids produce a range of 4-pregnen-3-ones and 4,6-pregnadien-3-ones as major defensive steroids but these compounds all possess certain physico-chemical properties that place them among the more highly anesthetic steroids known. The toxicity of these steroids presumably results from the depression of the respiratory center of the brain.

The gyrinid norsesquiterpenes apparently act in several ways. Gyrinidal and gyrinidione are highly toxic to fish but possess little definitive anesthetic quality. Judged from the hemorrhaging that takes place in minnows treated with these compounds, they may be membrane active, i.e., possibly hemolytic agents. Gyrinidone possesses anesthetic qualities similar to those of the steroids. To our knowledge, the anesthetic quality of oxygenated norsesquiterpenes has not previously been recognized. Further investigation of gyrinidione and its analogs could lead to the discovery of compounds that would be useful medicinally.

When minnows are placed in concentrated aqueous solutions of steroids where the rate of steroid absorption is far more rapid than deactivation mechanisms, many steroids apparently have similar activities suggesting that they may exert their effects via a very nonspecific process, possibly membrane stabilization (Seeman, 1969). However, certain limits on the physiochemical properties of steroids do govern activity, as not all steroids are active against fish. Water solubility appears to be of great importance in determining the activity of steroids administered in solution to fish. Other properties such as the partition coefficient of steroids across the aqueous–lipid interface of membranes may be of even greater importance. Until the actual rates of uptake of steroids into fish are measured it is impossible to state whether the

steroids found to be inactive in minnow bioassays appear so because they fail to reach the target tissues or whether they are innately less toxic even though they gain access to the cells of the body.

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