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NORSESQUITERPENES AS DEFENSIVE TOXINS OF WHIRLIGIG BEETLES (COLEOPTERA: GYRINIDAE)

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Abstract—By use of a minnow bioassay, toxins were detected in the pygidial secretions of the gyrinid beetles *Dineutus assimilis* (Kirby) and *Dineutus nigrior* Roberts. The active agents, which may be largely responsible for the relative immunity of the Gyrinidae from predation, were isolated and identified as the norsesquiterpenes *gyrinidione* [(E)-1-methyl-2-formyl-3-(1′-methylhex-3′-ene-2′,5′-dione)-cyclopentane], *gyrinidone* [(E,Z)-2-hydroxy-5,9-dimethyl-4-(but-1′-ene-3′-one)-3-oxo-bicyclo[4.3.0]-non-4-ene], *gyrinidal* [(E,E,E)-3,7-dimethyl-8,11-dioxo-2,6,9-dodecatrienal], and *isogyrinidal* [(E,E,Z)-3,7-dimethyl-8,11-dioxo-2,6,9-dodecatrienal]. Since gyrinidione and isogyrinidal are being reported for the first time, their physical and chemical properties are presented and biosynthetic relationships of the four norsesquiterpene structures are discussed. About 50% of the beetle defensive material was norsesquiterpenes, 25% polar lipids, and 20% could not be extracted from water into chloroform. As quantified by gas-liquid chromatography, *D. assimilis* contained 245 ± 73 μg and *D. nigrior* 144 ± 64 μg norsesquiterpenes per individual. The average relative composition of norsesquiterpenes in the pygidial secretions of both beetle species was constant: isogyrinidal, 6%; gyrinidone, 7%; gyrinidione, 36%; and gyrinidal, 48%. When administered externally in solution to fish, isolated norsesquiterpenes possessed narcotic and toxic activity similar to that of the anesthetic steroids deoxycorticosterone (DOC) and testosterone. Minnow dose-response curves demonstrated that gyrinidione and gyrinidal (LC_{100} = *ca.* 2 μg/ml) were as toxic to fish as was DOC (LC_{100} = *ca.* 3 μg/ml). Gyrinidone was less toxic (LC_{100} = *ca.* 15 μg/ml) while isogyrinidal was relatively inactive (LC_{100} = *ca.* 90 μg/ml).

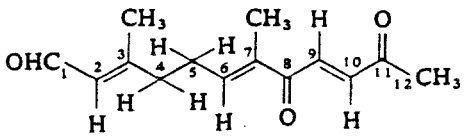
Key Words—biocommunication, defensive secretions, Gyrinidae, norsesquiterpenes, gyrinidal, isogyrinidal, gyrinidone, gyrinidione, steroid anesthesia.

INTRODUCTION

Adult whirligig beetles, family Gyrinidae, commonly inhabit the surface of ponds, small lakes, and some slow-flowing streams. Being gregarious, these beetles frequently assemble into stable clusters and at times congregate by the thousands into compact flotillas. Although they seemingly represent a food source conspicuously available to predators both from above and below the water surface, the relative immunity of gyrinids from predation by birds, amphibians, and fish has been amply documented (Wilson, 1923; Benfield, 1970 and references therein).

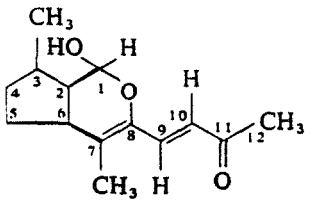
Previously, gyrinid immunity from predation has been attributed largely to morphological adaptations for escaping predators such as visually acute compound eyes adapted for vision both in air and in water (Leech and Chandler, 1956), a well developed Johnston's organ sensitive to minute disturbances in water (Rudolph, 1967), and a powerful swimming apparatus (Natchtigall, 1965). Although it had long been a matter of speculation (Wilson, 1923), the odorous secretions from the paired pygidial glands of the Gyrinidae have only recently been shown to have defensive value. In a series of feeding experiments, Benfield (1972) clearly demonstrated that gyrinid pygidial secretions served as feeding deterrents to several species of fish and to the common newt. Thus, in addition to adaptations for escape, chemical agents have been implicated as principal regulators of gyrinid predator-prey interactions.

The pygidial secretions of the Gyrinidae have attracted the attention of several groups of chemists who have identified some of the components from various species. Schildknecht and Neumaier (1970) partially characterized a major component of the pygidial secretions of *Gyrinus natator* as a C_{14} tricarbonyl compound with the molecular formula $C_{14}H_{18}O_3$ and Schildknecht et al. (1972*a*) established the complete structure of this component as the norsesquiterpene (E,E,E)-3,7-dimethyl-8,11-dioxo-2,6,9-dodecatrienal, *gyrinal* (Structure I),



(I)

and reported its presence in *Gyrinus substriatus* and *Gyrinus minutus* as well. Meinwald et al. (1972) independently identified (I), which they named *gyrinidal*, from *Gyrinus ventralis*, *Dineutus hornii*, and *Dineutus serrulatus* and mentioned that at submicrogram levels this material was a feeding deterrent to a species of bass. The cyclopentanoid norsesquiterpene *gyrinidone* (II),



(II)

was identified by Wheeler et al. (1972) as the major component of the pygidial secretions of *Dineutus discolor* and the presence of a mixture of unidentified isomeric aldehydes was noted. The volatile odorous components of *G. natator* pygidial secretions have been identified as 3-methylbutyraldehyde and 3-methylbutan-1-ol (Schildknecht et al., 1972*b*).

Although a number of compounds have been identified from the pygidial secretions of the Gyrinidae, their biological activities have received little or no study. Some of these compounds may be primarily responsible for the repellent nature of unfractionated pygidial secretions (Meinwald *et al.*, 1972). Others may function as the water-borne gyrinid alarm substances also present in these secretions (Benfield; 1970). The identified components, as well as other unidentified pygidial agents, may possess still further biological activity.

In this study, the pygidial secretions of *Dineutus assimilis* (Kirby) and *Dineutus nigrior* Roberts were examined for agents possessing narcotic or toxic activity similar to that of the dytiscid defensive steroids (Schildknecht, 1970; Miller and Mumma, 1973). Such toxins were detected by a minnow bioassay and were isolated and identified as gyrinidal, gyrinidone, and two new norsesquiterpenes, *gyrinidione* and *isogyrinidal*. Quantitative data on the composition of pygidial secretions are presented and a comparison is drawn between the biological activity of the norsesquiterpenes and that of the anesthetic steroids.

METHODS AND MATERIALS

Collection of Beetles and Pygidial Secretions

D. assimilis and *D. nigrior* were netted by the thousands from a large irrigation reservoir at the Rock Springs experimental farms 10 miles southwest of State College, Pennsylvania. Beetle collection was greatly facilitated by use of a flotation procedure, employing a chain of logs fastened end to end, that forced the gyrinids into large assemblages near the shoreline.

The electrical shocking procedure for mass collection of water beetle defensive secretions (Miller and Mumma, 1973) was used to efficiently collect the white, paste-like pygidial secretions in aqueous solution. By shocking 500 beetles (50–100 at a time) in the same 500-ml sample of tap water, opaque milky-white suspensions of defensive materials were obtained. Aqueous

defensive suspensions that were not immediately fractionated were stored at 8°C in sealed flasks.

Bioassay for Toxicity

The bioassay animals, fathead minnows (*Pimephales promelas* Raf.) weighing between 1 and 2 grams, were purchased from a live-bait dealer and were available year-round. The physiological state of all batches of minnows was standardized by storing them in aquaria at 5°C without feed for at least 48 hours before they were used for bioassays. As needed, minnows were allowed to equilibrate from 5°C to 25°C over a period of 2–3 hr and were subsequently used for bioassays within the next few hours. All bioassays were carried out in a temperature bath at 25 ± 0.5°C. Maximal sensitivity and excellent reproducibility of the bioassay were realized when these procedures were followed.

In initial tests for toxicity of gyrinid pygidial agents, the secretions of at least 10 beetles were added to 50 ml of well aerated water containing 3 *P. promelas*. Immediately upon addition of these materials the minnows characteristically reacted violently, darting about the 250-ml beakers in which they were contained, occasionally leaping into the air. After several minutes in the defensive solutions, the swimming ability of minnows was impaired and they suffered loss of equilibrium, turning on their sides when not actively swimming. Muscular activity progressively diminished, culminating in death. Minnow survival time in solutions of defensive secretions was dependent upon the concentration of pygidial agents administered. This simple bioassay procedure was used to trace the toxic factors as the pygidial materials were fractionated by standard chemical procedures.

Dose-responses curves for the isolated toxins and several steroid standards were obtained by recording the average elapsed time from the addition of varying amounts of the pure compounds to 3 *P. promelas*, contained in 50 ml of aerated tap water in a 250-ml beaker, until the gill-pumping motions of the fish ceased. Minnows used in these experiments averaged 1.2 g (range 1.0–1.5 g). Stock solutions of the compounds to be tested were prepared in ethanol (66.7 μg/μl) and the appropriate amount of stock solution was injected into the bioassay containers. Appropriate control experiments demonstrated no additive toxic effects from ethanol. All compounds tested had solubilities greater than 120 μg/ml water.

Isolation of Toxic Agents

The defensive suspensions of *D. assimilis* and *D. nigrior* were thrice extracted with equal volumes of chloroform and the resultant fractions bio-

assayed. Aliquots of the lipid portion of the defensive solutions, dissolved in 20 μl ethanol, produced toxic effects resembling those caused by the original suspensions while the aqueous fraction was completely inactive.

When chloroform extracts of the defensive secretions were further fractionated by silicic acid column chromatography (CC) into nonpolar (eluted in $CHCl_3$) and polar (eluted in CH_3OH) lipids (Rouser et al., 1965), only the nonpolar lipids were toxic. Nonpolar lipids were fractionated by thin-layer chromatography (TLC) using the adsorbents Supelcosil 12A and 12B, containing a zinc silicate phosphor (Supelco, Inc., Bellefonte, Pennsylvania) and the solvent systems diethyl ether–petroleum ether (60–70°C) (50:50, v/v) and cyclohexane–ethyl acetate (50:50, v/v). Chromatograms were examined for bands under long- and short-wavelength ultraviolet (UV) light and by charring with H_2SO_4. All resultant bands were scraped, eluted from the adsorbent with chloroform–methanol (2:1), and bioassayed. Active materials were isolated in quantities sufficient for chemical identifications and further bioassay studies by preparative TLC, employing multiple development in the above-mentioned solvent systems, and by extensive column chromatography (CC) on Supelcosil-ATF 120 (Supelco, Inc., Bellefonte, Pennsylvania), eluting with increasing percentages of ethyl acetate in cyclohexane or increasing percentages of chloroform in benzene.

Instrumentation for Identification of Isolated Toxins

UV spectra were recorded on a Bausch and Lomb Spectronic 600 recording spectrophotometer. Infrared (IR) spectra were recorded on a Perkin-Elmer 621 IR spectrophotometer using a focusing-beam condenser and a 0.4-μl capacity microcell containing the compounds in chloroform solution. Low-resolution mass spectra were obtained on a LKB-9000 gas-liquid chromatograph (GLC) interfaced mass spectrometer using a 6-ft × $\frac{3}{16}$-in O.D. glass column packed with 2.0% OV-1 on Supelcoport 100/120 (Supelco, Inc., Bellefonte, Pennsylvania), a He flow of 30 ml/min, and a column oven temperature of 180°C. High-resolution mass spectra were taken on an AEI Model MS-902 mass spectrometer using a direct sample inlet system. All mass spectra presented in the text were recorded at an ionization potential of 70 eV. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian A-60A NMR spectrometer.

GLC Analysis

The nonpolar lipid components of the gyrinid defensive suspensions were chromatographed on a MicroTek 220 gas-liquid chromatograph using a 6-ft × 4-mm I.D. glass column packed with 2.0% OV-1 on Supelcoport 100/120, a

N_2 flow of 40 ml/min and a column oven temperature of 170°C. Metal columns were avoided since they catalyzed isomerizations of the defensive compounds. The dual-flame ionization detector signal was coupled to an Aerograph 471 digital integrator for recording peak areas.

Since absolute identification of *D. assimilis* and *D. nigrior* requires microscopic examination of their genitalia, separation of the two species could not be accomplished without loss of some defensive materials due to manipulation. Therefore, in quantifying the defensive titer of these beetles it was necessary to first separately collect the pygidial secretions of each individual in the sample and then make identifications. Pygidial secretions of individual beetles were collected in 2 ml tap water by electrical shocking, then immediately extracted into 2 ml chloroform and quantified by GLC.

RESULTS

Yields from Fractionation of Defensive Suspensions

Table 1 presents the dry-weight composition of *D. assimilis* pygidial secretions upon fractionation. The major portion of the secretions (81%) including all of the toxic agents, was extractable from water into chloroform. A significant portion of the secretions (23%) was polar lipids which had no adverse effects on *P. promelas* when bioassayed. Toxic compounds constituted nearly the entire nonpolar lipid fraction and were isolated in amounts averaging nearly 50% of the starting dry weight of 0.6 mg per beetle. Relative

TABLE 1. FRACTIONATION OF THE PYGIDIAL SECRETIONS OF *Dineutus assimilis*[a]

Fraction	Toxic to fish	Dry weight (μg/beetle)	Percent of total
Aqueous defensive suspension[b]	+	602	100
Water layer after chloroform[b] extraction	−	98	16
Chloroform extract	+	490	81
Polar lipids	−	140	23
Neutral lipids	+	320	53
Bands from TLC	+	300	50

[a] Typical yields from fractionation of the excretions of 1000 beetles.
[b] Weight of residue from tap water subtracted.

yields for *D. nigrior* were similar although actual quantities of each fraction were consistently less.

TLC of Nonpolar Lipids

TLC of the nonpolar lipid portions of fresh pygidial secretions from either beetle species resolved four distinct bands, all toxic in some degree to *P. promelas*. In the solvent system diethyl ether–petroleum ether (60–70°C) (50:50, v/v), the toxic bands possessed R_f values of 0.10, 0.20, 0.44, and 0.58 and are designated Bands A–D, respectively. Bands B and D were the predominant components while Bands A and C were minor components. The eluate of pooled portions of chromatograms exclusive of Bands A–D was

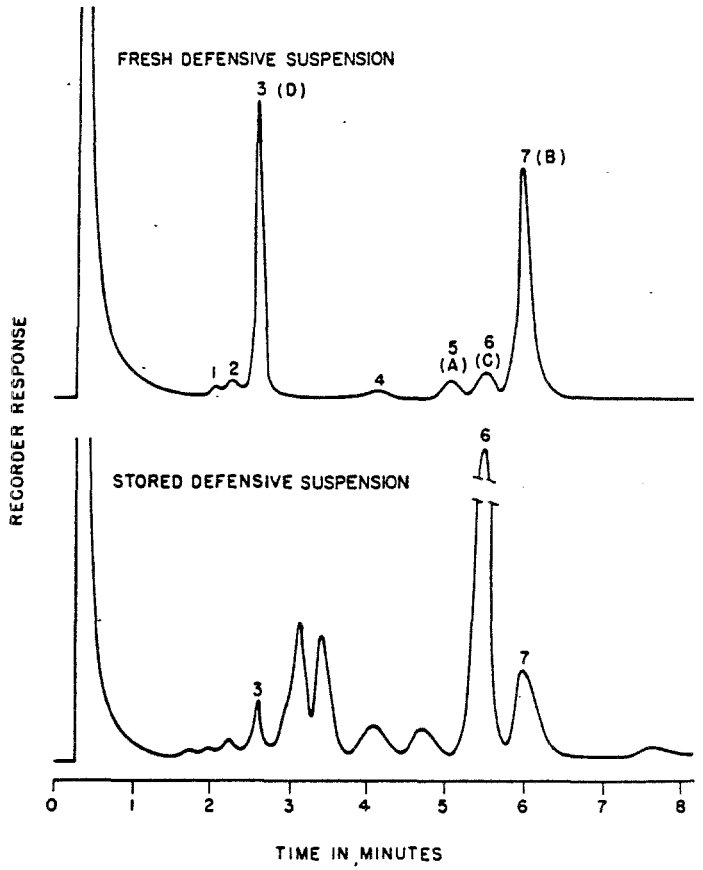


FIG. 1. Representative gas-liquid chromatograms of the chloroform extracts of fresh and stored pygidial secretions of *Dineutus assimilis* and *Dineutus nigrior*. Letters represent corresponding TLC Bands.

not toxic to *P. promelas*, although traces of further components were detected.

Numerous bands resulted from TLC of the nonpolar lipid portion of stored defensive suspensions, the mixture being so complex that compounds could not be completely resolved. The material in greatest quantity was Band $\ddot{\ddot{C}}$, which in fresh secretions was a minor component.

The chromatographic mobility of the toxic agents in various solvent systems was similar to that of testosterone and deoxycorticosterone (DOC), steroids which are representatives of the narcotizing agents from the prothoracic defensive glands of the Dytiscidae (Schildknecht, 1970; Miller and Mumma, 1973). When chromatograms were stained with I_2 or sprayed with 2,4-dinitrophenylhydrazine reagent (Stahl, 1969), Bands A–D developed a more intense color than did equivalent amounts of monounsaturated monocarbonyl standards. A positive response to potassium ferricyanide–ferric chloride spray reagent by all four of these compounds indicated that they all possessed some enol character.

GLC of Fresh and Stored Defensive Materials

Gas-liquid chromatograms of the nonpolar lipid portions of fresh and stored defensive materials (Figure 1) clearly demonstrated compositional changes upon storage. Freshly collected defensive secretions yielded seven distinct peaks with Peaks 3 and 7 making up over 80% of the total. Temperature programming starting at ambient temperature and progressing to 300°C resolved only traces of additional compounds. Numerous unresolved peaks appearing in gas chromatograms of the nonpolar lipid portions of stored defensive suspensions, demonstrated degradation of the original compounds with major changes in relative composition. Upon storage, the relative percentages and absolute quantities of Peak 3 and 7 greatly diminished while the relative percentage of Peak 6 greatly increased. The actual quantities of Peak 6 increased from less than 20 μg per beetle in fresh secretions to more than 100 μg per beetle in stored secretions, indicating that some of the original compounds were apparently being converted into Peak 6.

Characterization and Identification of Toxic Compounds

TLC Band B (GLC Peak 7), isolated by CC from fresh defensive secretions cochromatographed with an authentic sample of gyrinidal in various TLC solvent systems and on several GLC columns. Band B demonstrated UV λ_{max}^{EtOH} 238 nm (ε = 25,000); a mass spectrum identical to authentic gyrinidal, both giving prominent mass spectral peaks (relative abundance calculated from *m/e* 43 as base peak) at *m/e* 234 (3, M), 216 (6), 206 (3), 205 (4), 191 (7), 173 (17), 149 (23), 145 (14), 135 (17), 125 (21), 109 (62), 98 (24), 95 (25), 93 (18), 91 (31), 79 (30), 67 (24), 55 (42), and 43 (100); NMR ($CDCl_3$, δ in ppm

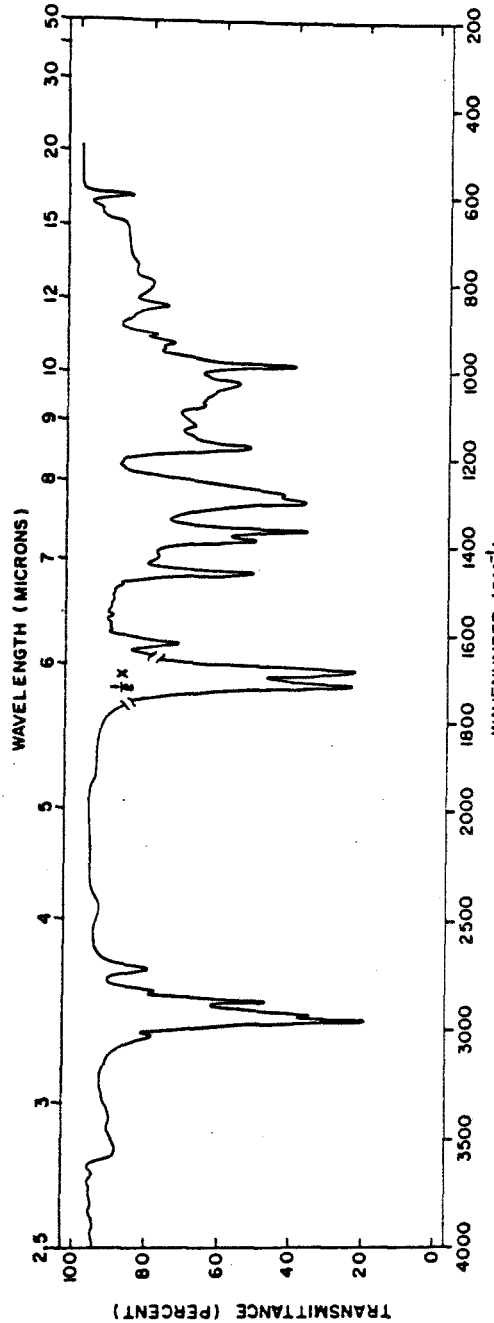


FIG. 2. IR spectrum ($CHCl_3$ solution) of TLC Band D (gyrinidione) from *Dineutus assimilis*.

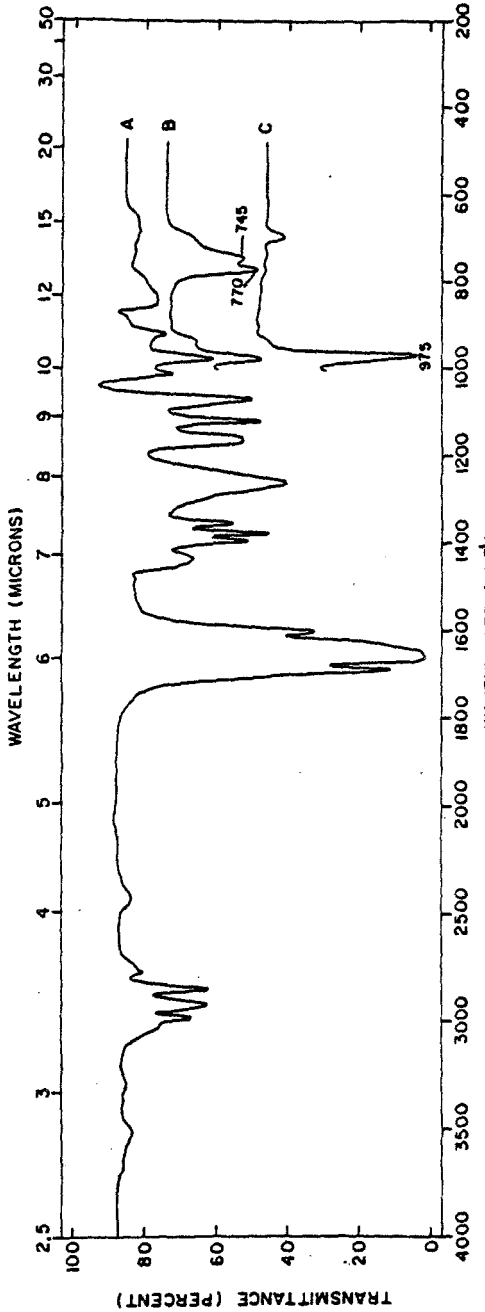


FIG. 3. IR spectrum of TLC Band A (isogyrinidal) from *Dineutus assimilis*. A = Band A in $CHCl_3$ solution; B = Band A in CS_2 solution; C = gyrinidal in CS_2 solution.

relative to internal tetramethylsilane) 1.89 (d, J = 1 Hz, 3 H), 2.24 (d, J = 1.5 Hz, 3 H), 2.38 (s, 3 H), 2.4–2.7 (m, 4 H), 5.90 (d, J = 8 Hz, 1 H), 6.5–6.8 (m, 1 H), 6.85 (d, J = 16 Hz, 1 H), 7.42 (d, J = 16 Hz, 1 H), 9.98 (d, J = 8 Hz, 1 H). Since these data are consistent with those collectively reported for gyrinidal (Schildknecht et al., 1972*a*; Meinwald et al., 1972; Miller et al., 1973), the compound represented by TLC Band B was assigned structure I, gyrinidal.

TLC Band C (GLC Peak 6), isolated in high yield from stored defensive suspensions by CC, was crystallized out of aqueous methanol and had a mp of 81–83°C. It demonstrated UV λ_{max}^{EtOH} 318 nm (ε = 13,300) and 234 (ε = 8,700); prominent mass spectral peaks (relative abundance calculated from *m/e* 43 as base peak) at *m/e* 236 (17, M), 218 (6), 207 (10), 175 (19), 151 (35), 125 (32), 111 (26), 109 (43), 98 (50), 81 (37), 55 (36), 43 (100); NMR ($CDCl_3$) δ 1.10 (d, J = 5 Hz, 3 H), 1.86 (d, J = 1 Hz, 3 H), 2.28 (s, 3 H), 4.50 (broad s, 1 H), 5.02 (d, J = 5 Hz, 1 H), 6.48 (d, J = 16 Hz, 1 H), 7.33 (d, J = 16 Hz, 1 H), and seven additional protons between 1.1 and 2.9. Since these data are in close agreement with those reported by Wheeler et al. (1972) for gyrinidone, the compound represented by TLC Band C was ascertained to be gyrinidone (II). A higher UV absorption coefficient was determined for gyrinidone than was previously reported; however, this is probably explained by the fact that our product was crystalline.

TLC Band D (GLC Peak 3), isolated by CC from fresh defensive suspensions possessed UV λ_{max}^{EtOH} 230 nm (ε = 11,200). Its IR spectrum (Figure 2) contained prominent absorptions bands (listed in cm^{-1}) indicating, among others, these structural features; 3040 (—C=C—H stretch), 2730 (aldehydic C—H stretch), 1720 (aldehydic C=O stretch), 1680 (—C=C—C(=O)—R carbonyl stretch), 1360 (—C(=O)—CH_3 methyl bending), 981 (*trans* —CH=CH— bending). High-resolution mass spectrometry demonstrated a molecular formula of $C_{14}H_{20}O_3$ (mass per unit charge [*m/e*] 236.1437, calculated 236.1412). Band D gave the following low-resolution fragmentation pattern (relative abundance calculated from *m/e* 151 as base peak and *m/e* assignments from high resolution); *m/e* 236 (3, $C_{14}H_{20}O_3$, M), 221 (2, $C_{13}H_{17}O_3$, M—CH_3), 218 (7, $C_{14}H_{18}O_2$, M—H_2O), 208 (7, $C_{13}H_{20}O_2$, M—CO), 207 (8, $C_{12}H_{15}O_3$, M—C_2H_5), 193 (4, $C_{12}H_{17}O_2$, M—$COCH_3$), 190 (5, $C_{13}H_{18}O$), 189 (7, $C_{13}H_{17}O$), 175 (10, $C_{12}H_{15}O$), 168 (14, $C_9H_{12}O_3$), 165 (20, $C_{11}H_{17}O$), 151 (100, $C_{10}H_{15}O$), 135 (18, $C_9H_{11}O$), 125 (25, $C_7H_9O_2$), 109 (34, C_7H_9O), 93 (23, C_7H_9), 83 (30, C_5H_7O), 69 (15, C_4H_5O), 55 (52, C_3H_3O, C_4H_7), 43 (71, C_2H_3O). The NMR absorptions of Band D are shown in Table 2.

TABLE 2. NMR SPECTRUM OF TLC BAND D (GYRINIDIONE) FROM *Dineutus assimilis*

δ (in $CDCl_3$)	Number of protons	Splitting pattern
1.10	3	d, J = 6 Hz
1.17	3	d, J = 7 Hz
1.2–2.7	7	complex
2.37	3	s
2.7–3.3	1	m
6.89	2	s
9.79	1	broad filled in doublet, J = 3 Hz
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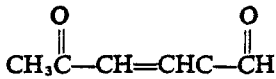
	20	

The spectral data of I and II as well as that of 3-hexene-2,5-dione (III)

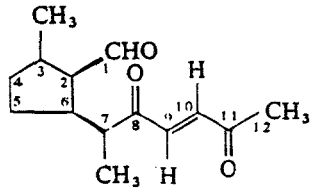


(III)

(Sugiyama and Kashima, 1970) were useful in structural establishment of Band D. In the NMR spectrum of *trans* III, the equivalent *trans* vinylic protons appear as a sharp singlet at δ 6.78 in $CDCl_3$, and the methyl ketones appear as a singlet at 2.38. The UV λ_{max}^{EtOH} of *trans* III is 228 nm (ε = 14,000). Corresponding values for *cis* III are δ 6.30, 2.29, and UV λ_{max}^{EtOH} 224 nm (ε = 8,600).

Presence of a methyl ketone in Band D was evidenced by the three-proton NMR absorption at δ 2.37, which corresponded to the methyl ketone absorptions of I and *trans* III (both 2.38), the prominent MS peak at *m/e* 43, and IR absorption at 1360 cm^{-1}. The sharp two-proton singlet at δ 6.89, the *trans* —CH=CH— IR absorption at 981 cm^{-1}, and the UV λ_{max}^{EtOH} 230 nm (ε = 11,200) all corresponded closely to the spectral data of *trans* III and indicated a symmetrical *trans* ene-dione system in Band D. While the aldehydic proton of the α,β unsaturated aldehyde of I appeared at δ 9.98 as a doublet (J = 8 Hz), the aldehydic proton of Band D appeared further upfield as a doublet at δ 9.79 (J = 3 Hz), suggesting a saturated aldehyde. The three-proton absorptions at δ 1.10 and 1.17 corresponded closely to the C-3 methyl of II (1.10) and were interpreted as methyls attached to tertiary carbon atoms. The eight additional protons detected in the NMR spectrum of Band D gave no clear splitting patterns and appeared to be aliphatic or alicyclic.

Incorporation of the methyl and saturated aldehyde moieties into Band D, while complying with the restrictions of only two olefinic protons and a molecular formula of $C_{14}H_{20}O_3$, was achieved by establishing a cyclopentanoid ring as in II. Although they possess similar functional groups, the retention time of Band D on OV-1 (Figure 1) was less than half that of I, sup-



(IV)

porting cyclization of Band D. On the basis of these data we propose structure IV, *gyrinidione*, as the compound represented by TLC Band D.

The broad filled-in doublet of the aldehydic proton absorption (C-1) can be explained by virtual coupling of the C-2, C-3, and C-6 protons (Silverstein and Bassler, 1967). The remaining methyl (δ 1.17) was assigned to C-7 since a methyl branch occurred at an analogous position in both gyrinidal and gyrinidone.

It becomes obvious that II and IV should be interconvertible by formation or opening of a ring involving a hemiacetal linkage between the aldehyde and the C-8 ketone. Confirmation of structure IV was achieved and a biosynthetic relationship to II was demonstrated by the conversion of IV to II. Gyrinidione dissolved in aqueous acetic acid (pH 3) for four days at room temperature, in darkness and under N_2, yielded a product (35% conversion) that had TLC, GLC, and mass spectral properties identical to II. The stereochemistry of IV at C-2 and C-6 was therefore assigned analogous configurations to those offered by Wheeler et al. (1972) for II.

TLC Band A (GLC Peak 5), isolated by preparative TLC from fresh defensive secretions, was readily interconvertible with I. Gyrinidal, upon 24 hours exposure to light while on silica gel thin-layer chromatogram, gave rise to Band A (yields >20%). Band A was converted to I in pyridine–acetic

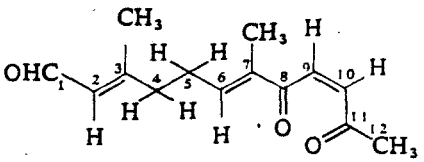
TABLE 3. NMR SPECTRUM OF TLC BAND A (ISOGYRINIDAL) FROM *Dineutus assimilis*

δ ($CDCl_3$)	Number of protons	Splitting pattern
1.83	3	d, J = 1 Hz
2.17	3	d, J = 1 Hz
2.23	3	s
2.2–2.6	4	m
5.87	1	d, J = 8 Hz
6.2–6.6	1	m
6.37	1	d, J = 12 Hz
6.58	1	d, J = 12 Hz
9.97	1	d, J = 8 Hz
	18	

anhydride (3:2) at 60°C for 0.5 hr (products identified by GLC–MS). Partial conversion of Band A to I occurred in both pyridine–acetic anhydride (3:2) at 22°C and in pyridine alone at 60°C.

The similar spectral properties of Band A and I suggested they were isomers. Band A demonstrated λ_{max}^{EtOH} 237 nm (ε = 20,500); a mass spectrum (M = 234) identical in all respects to that of I; and an IR spectrum (Figure 3) very similar to that reported by Schildknecht and Neumaier (1970) for I. Although the majority of the IR absorption bands of I and Band A were identical, a difference was detected in the region from 1000 to 650 cm^{-1} when the IR absorption of equivalent amounts of these compounds was recorded in CS_2 (Figure 3). While both compounds showed an absorption band at 975 cm^{-1} (*trans* —CH=CH— bending), I absorbed more strongly than did Band A. The characteristic *cis* absorption bands occurring at 745 and 770 cm^{-1} in the spectrum of Band A were lacking in the CS_2 spectrum of I. Similar absorptions are reported for the *cis* double bond of maleic acid (765, 785 cm^{-1}), *cis*-2,5-dimethoxy-2,5-dimethyl-2,5-dihydrofuran (770 cm^{-1}), and 15-*cis*-β-carotene (780 cm^{-1}).

The NMR absorptions of Band A (Table 3) showed the presence of 18 protons with a resonance pattern very similar to I. Despite the striking similarities of the NMR spectra of I and Band A, some differences in chemical shifts and splitting patterns of corresponding resonances gave reason to propose that Band A is the C-9 *cis* isomer of I, (E,E,Z)-3,7-dimethyl-8,11-dioxo-2,6,9-dodecatrienal, *isogyrinidal* (V).



(V)

NMR assignments were made as follows: δ 1.83 (methyl of C-7), 2.17 (methyl of C-3), 2.23 (methyl of methyl ketone), 2.2–2.6 (methylene protons of C-4, C-5), 5.87 (vinyl proton of C-2), 6.2–6.6 (vinyl proton of C-6), 6.37, 6.58 (*cis* vinyl protons of C-9, C-10), 9.97 (aldehydic proton). The upfield shift of the methyl ketone absorption of V (δ 2.23) in relation to the methyl ketone of I and IV (δ 2.38) corresponds to the upfield shift of the methyl ketone absorption incurred in the transition of *trans* III (δ 2.38) to *cis* III (δ 2.29). An upfield shift in the center of the AB quartet pattern of the C-9, C-10 vinyl doublets of V (δ 6.45) was realized relative to I (δ 7.15). A similar upfield shift occurs in the vinyl proton absorption in the transition from *trans* III (δ 6.78) to *cis* III (δ 6.30). The reduction of the coupling constant of the C-9, C-10 vinylic protons from 16 Hz in I to 12 H_z in V also suggests a transition from

trans to *cis*. The other slight differences between the NMR spectra of V and I are consistent with this isomerization and suggest no further differences. Presence of the *cis* ene-dione system in V was confirmed by chelate formation with ferric chloride. When TLC chromatograms of gyrinid norsesquiterpenes were sprayed with 5% ethanolic ferric chloride, isogyrinidal formed a brick-red chelate while the other norsesquiterpenes did not.

GLC Quantification of Nonpolar Lipid Components

The relative composition of the nonpolar lipid portions of *D. assimilis* and *D. nigrior* pygidial secretions are presented in Table 4, and absolute quantities of compounds are shown in Table 5. Although the mean deviations show considerable individual variation, the average relative composition of the major norsesquiterpene components was surprisingly constant when gyrinid species and sexes were compared. Toxins constituted at least 98% of the detected compounds since Peaks 3, 5, 6, and 7 were all toxic to *P. promelas*.

The norsesquiterpene defensive titer of the two gyrinid species differed (Table 5). *D. assimilis* contained an average of 245 ± 73 μg norsesquiterpenes per individual while *D. nigrior*, the larger beetle, contained 144 ± 64 μg per individual (difference significant, $P = 0.01$). *D. assimilis* females contained a significantly higher defensive titer than did males ($P = 0.01$); however, no such difference was observed between the sexes of *D. nigrior*. As indicated by the large mean deviations, individual defensive titers were quite variable. The *D. assimilis* norsesquiterpene defensive titer ranged from 86 to 450 μg per individual and that of *D. nigrior* ranged from 51 to 317 μg per individual.

TABLE 4. RELATIVE COMPOSITION OF THE CHLOROFORM EXTRACTS OF PYGIDIAL SECRETIONS FROM INDIVIDUAL *Dineutus assimilis* AND *Dineutus nigrior* AS QUANTIFIED BY GLC

Species	Sex	Number of individuals examined	Relative percentage of GLC peaks[a]				
			1	3	5	6	7
D. assimilis	♂	18	1.3 ± 0.9[b]	36.9 ± 6.0	6.5 ± 1.9	6.6 ± 2.3	47.5 ± 18.0
D. assimilis	♀	8	0.8 ± 0.3	35.1 ± 5.3	7.6 ± 0.8	7.6 ± 3.4	48.0 ± 9.7
D. nigrior	♂	4	3.2 ± 3.1	36.2 ± 7.7	6.3 ± 0.5	6.8 ± 1.6	46.8 ± 4.4
D. nigrior	♀	9	1.0 ± 0.6	36.4 ± 4.8	4.4 ± 0.9	8.7 ± 4.0	48.1 ± 4.9

[a] GLC peaks numbered as in Figure 2, Peak 3 = gyrinidione, 5 = isogyrinidal, 6 = gyrinidone, 7 = gyrinidal; minor peaks not listed.

[b] Mean deviation.

TABLE 5. COMPOSITION OF THE CHLOROFORM EXTRACTS OF PYGIDIAL SECRETIONS FROM INDIVIDUAL *Dineutis assimilus* AND *Dineutus nigrior* AS QUANTIFIED BY GLC

Species	Sex	Number of individuals examined	Avg. body weight per individual (mg)	Average quantity of compound per individual (μg)					
				GLC peak[a]					Total compounds[b]
				1	3	5	6	7	
D. assimilis	♂	18	69.6±6.0[c]	3.3±3.0	80.9±22.7	14.3±5.9	17.0±9.5	99.7±29.2	217.1±58.8
D. assimilis	♀	8	74.7±5.0	3.3±1.1	110.1±24.9	27.5±8.3	27.6±13.5	136.5±30.1	308.9±73.2
D. nigrior	♂	4	93.0±4.5	3.2±1.8	58.2±24.6	10.6±5.7	10.8±6.1	71.3±25.8	158.4±61.1
D. nigrior	♀	9	92.9±4.0	1.8±1.9	48.9±26.7	6.3±3.4	13.5±9.4	61.1±24.5	138.8±60.0

[a] GLC peaks numbered as in Figure 2, Peak 3 = gyrinidione, 5 = isogyrinidal, 6 = gyrinidone, 7 = gyrinidal; minor peaks not listed.
[b] Summation of all GLC-detected compounds.
[c] Mean deviation.

Effects of Norsesquiterpenes and Steroids on Fish

Although a few differences were recognized, gyrinidione, gyrinidal, gyrinidone, DOC, and testosterone affected fish similarly when administered at 30 μg/ml water. Each compound initially caused considerable hyperactivity in fish; however, the hyperactivity elicited by the norsesquiterpenes was more violent and prolonged than that elicited by the steroids. In solutions of the steroids, fish rapidly became lethargic, lost their ability to orient into water currents, and soon turned on their sides showing little muscular activity aside from gill-pumping motions. Respiratory motions progressively diminished and ceased. Death soon followed and was often accompanied by muscular tremors. Norsesquiterpene-treated fish also rapidly became lethargic, but they maintained their ability to swim about and to orient into water currents comparatively longer than did those treated with equivalent dosages of steroids. Although the effect was delayed, norsesquiterpenes at 30 μg/ml also caused an equilibrium loss in fish, deep narcosis, and death.

Upon reaching a state of steroid narcosis where they could no longer right themselves, minnows placed into fresh water remained deeply anesthetized for approximately an hour but usually recovered fully. Minnows lightly anesthetized with norsesquiterpenes could likewise recover. A deeper state of steroid-induced narcosis was tolerated by the fish than that induced by the norsesquiterpenes.

Toxicity of Norsesquiterpenes and Steroids

Toxicities of gyrinidione, gyrinidal, gyrinidone, DOC, and testosterone are shown in Figure 4 as a function of external concentration. Log × log plots of minnow survival time *vs.* external concentration of toxin resulted in a linear response at the higher range of concentrations. As concentration decreased, a point of inflection was reached below which further decreases in concentration resulted in disproportionately longer survival times. Shortly below this inflection point minnows began to survive the treatments. The lowest concentration of toxin lethal to all fish contained therein is by definition the LC_{100} and is approximated by the concentration at which each curve in Figure 4 becomes perpendicular.

Although the three norsesquiterpenes killed fish with nearly equal rapidity at concentrations of 30 μg/ml and above, gyrinidione and gyrinidal were considerably more toxic than gyrinidone at concentrations below 20 μg/ml. The estimated LC_{100} of gyrinidone was 15 μg/ml while that of gyrinidione and gyrinidal was only 2 μg/ml. Estimated LC_{100} values for the steroids were 3 μg/ml for DOC and 8 μg/ml for testosterone. Toxicities of gyrinidione, gyrinidal, and DOC were very similar throughout the range of concentrations

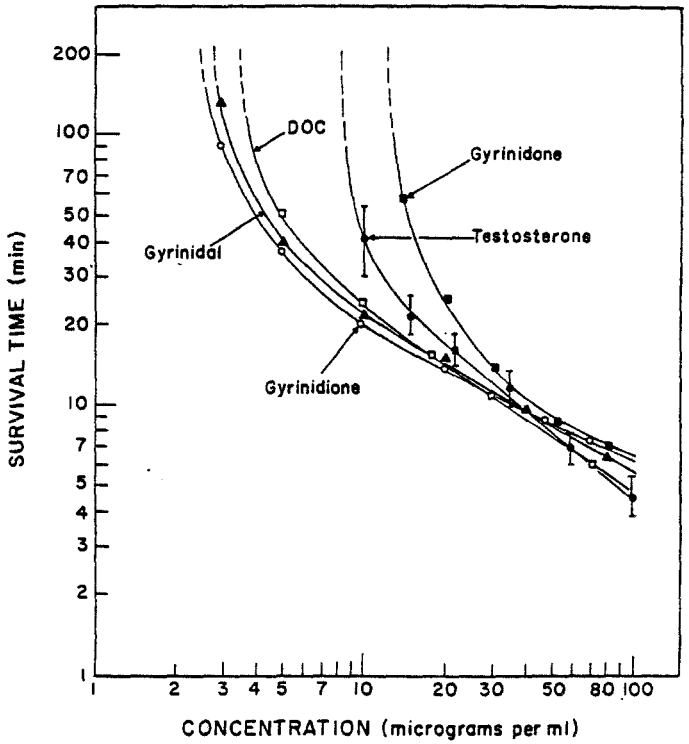


FIG. 4. Log × log plots of the survival time of *P. promelas* in 50-ml solutions of gyrinid norsesquiterpenes and several steroid standards. Standard deviations for testosterone are indicated by brackets and are representative of the deviations for the other compounds.

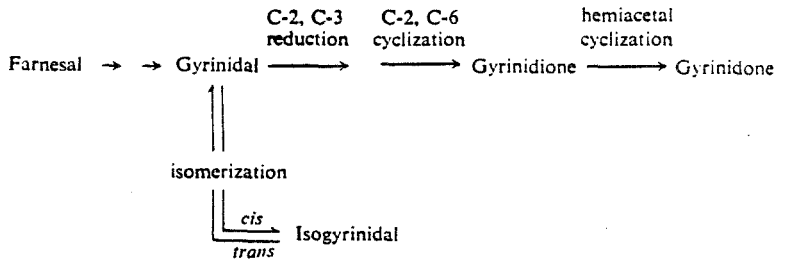
tested. Isogyrinidal was far less toxic than the other norsesquiterpenes requiring concentrations in the range of 70–90 μg/ml and exposures of over one hour to kill minnows.

In concentrations of norsesquiterpenes or steroids slightly lower than those that proved lethal, minnows were narcotized by both types of compounds, the latter inducing the deeper and more prolonged narcosis. Concentrations of these agents that were insufficient to cause such effects in minnows as equilibrium loss did cause them to become noticably lethargic. While remaining in sublethal concentrations of steroids or norsesquiterpenes minnows usually recovered completely from all effects within 3–5 hr, indicating that they could detoxify certain doses of these poisons. In general, if minnows survived for 3 hr in a given concentration of toxin administered in 50 ml water, they recovered from that treatment.

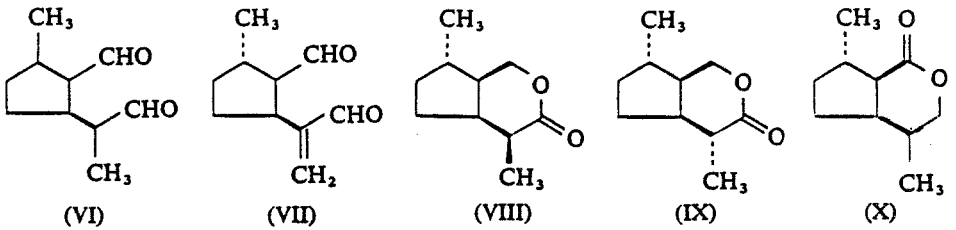
DISCUSSION

The pygidial glands of *D. assimilis* and *D. nigrior*, which occupy considerable space in the abdomens of these insects, secrete a complex mixture of components. Although at least 50% of the dry weight of pygidial secretions of these species was norsesquiterpenes, approximately 25% was characterized as polar-lipid and 25% as nonlipid. Paper chromatography of acid hydrolyzates of pygidial secretions demonstrated the presence of various amino acids, suggesting that some of the secretion is proteinacious. In water, gyrinid secretions form milky emulsions that can be broken by centrifugation or that settle out into a particulate layer upon storage. The non-norsesquiterpene portions of these secretions might serve important functions as adjuvants or spreaders of the norsesquiterpene toxins, or such components might themselves serve as defensive agents in a manner not reflected by the *P. promelas* bioassay.

In contrast to previous reports, *D. assimilis* and *D. nigrior* pygidial secretions contained more than one major norsesquiterpene component. Gyrinidal (48%) and gyrinidione (36%) were the major norsesquiterpene components of the secretions of these beetles but were found in combination with minor quantities of gyrinidone, isogyrinidal, and several unidentified trace components, some of which are isomers of the major components. Although we demonstrated that isogyrinidal slowly arises from gyrinidal upon exposure of the latter to light and that in acidic aqueous solution gyrinidione slowly gives rise to gyrinidone, chloroform extraction and GLC of gyrinid pygidial material immediately upon its excretion into water demonstrated the presence of both of these minor components. The average relative composition of norsesquiterpenes was identical (Table 4) in *D. assimilis* and *D. nigrior* and may reflect an established glandular equilibrium between norsesquiterpene components. One possible biosynthetic route by which these compounds may be related is represented by the following scheme.



A similar biosynthetic route starting from citral has been postulated and partially substantiated for the biosynthesis of the insect cyclopentanoid monoterpenes iridodial (VI), dolichodial (VII), iridomyrmecin (VIII), isoiridomyr-



mecin (IX) and the structurally related plant monoterpene nepetalactone (X) (Cavill and Robertson, 1965). The structural similarities of VI and VII to gyrinidione are striking, as are the structural similarities of VIII, IX, and X to gyrinidone.

The norsesquiterpene defensive toxins of *D. assimilis* and *D. nigrior* have limited chemical stability. Lengthy storage of these agents, their exposure to heat, air, pH changes, and especially light facilitate their degradation. Therefore, in establishing the glandular composition of these toxins, it is desirable to carry out the analyses as soon as possible after their collection. The presence of large amounts of gyrinidone and only minor quantities of gyrinidione and gyrinidal in stored aqueous suspensions of *D. assimilis* (Figure 1) was an artifact of the storage procedure. Future investigations on gyrinid defensive secretions should take the instability of the norsesquiterpenes into consideration.

Gyrinid pygidial secretions serve at least two defensive roles. As documented by Benfield (1972), pygidial secretions serve as potent feeding deterrents to predators. Although some predators initially ingested a beetle or two, they thereafter consistently rejected gyrinid beetles or food items to which the pygidial secretions were applied. As assayed by *P. promelas*, the norsesquiterpenes contained in the pygidial secretions are powerful toxins capable of serving a defensive role by poisoning predators. Under natural conditions aquatic organisms might contact enough beetle defensive agents by gill absorption to elicit behavioral responses; however, we doubt whether they would encounter sufficiently high external concentrations of these agents to be narcotized. It is more likely that predators would contact the defensive agents by ingesting whirligig beetles. Preliminary feeding experiments indicate that frogs (*Rana pipiens*) and fish (*Salmo gairdneri*) which are force-fed live whirligig beetles can contact enough defensive compounds to be stupefied or killed.

Although further feeding experiments are necessary to determine the oral toxicity of the norsesquiterpenes, an estimate of their toxicity to *P. promelas* when administered in external solution can be made from the data in Figure 4. For *P. promelas* in 50 ml of water the LC_{100} for gyrinidione and gyrinidal was slightly less than 3 μg/ml, with death occurring in approximately 2 hours. Since the 3 minnows in each beaker averaged 1.2 g, as much as 150 μg

of toxin could have been absorbed by the 3.6 g of fish or a reasonable estimate of the LD_{100} for gyrinidione and gyrinidal would be 42 μg/g fish. As calculated from Table 5, *D. assimilis* contains a combined average of approximately 210 μg of gyrinidione and gyrinidal per individual. Provided that absorption of these compounds from the digestive tract of a predator is reasonably rapid, it may take only a few consumed beetles to produce sufficient ill effects to communicate the message that their kind is an unsatisfactory food item.

One would expect that the effects of the beetle toxins would be dependent on the body weight of the predator. It follows that large fish and other large predators might be able to consume some whirligig beetles without suffering serious ill effects. The following observation related by Wilson (1923) supports such a contention. During the period of one summer, ponds containing immature bluegills, *Lepomis macrochirus*, were found to be highly populated by *D. assimilis* while an adjacent pond containing adult bluegills were kept free of these beetles. Examination of the stomach contents of the fish revealed that the young bluegills were consuming various aquatic beetles but no Gyrinidae. When some of the gyrinids were transferred to the pond containing adult bluegills, they were promptly eaten. Although this example demonstrates that some gyrinid adults are consumed by fish, only a few species are known to do so (Benfield, 1970). Among these are the bluegill fish and several species of bullheads. Extensive predation even by these species might be controlled by the defensive toxins.

Other fish species might just as well be able to consume gyrinids with slight ill effects, but they apparently do not. The repellent character of the pygidial secretions of gyrinids may largely preclude their being preyed upon by various predators large or small. Whether feeding patterns established when predators were small enough to be poisoned by gyrinid defensive toxins remain established in later life is a matter of speculation but may be a factor in gyrinid immunity from predation.

The observation that gyrinidal was repellent to fish (Meinwald et al., 1972) suggests that the toxic and repellent agents of gyrinid secretions may be one and the same. The distinction between the repellent and toxic nature of the norsesquiterpenes may be a matter of the dosage administered to the predator. Whether acting as repellents, toxins, or both, these agents undoubtedly play an important defensive role in the Gyrinidae, most likely in teaching predators to discriminate against whirligigs as an unsuitable food item. Examples of predators learning to avoid noxious prey, even when the effects of the defensive agents may be delayed, are discussed by Eisner (1970).

As assayed by fish, the physiological effects of the defensive norsesquiterpenes resemble those of the steroids. Both types of compounds have a protracted narcotizing action on fish that is lethal in high concentrations and reversible in low concentrations. DOC, testosterone, progesterone, and

various other steroid hormones common to vertebrates are recognized anesthetic agents that have a protracted narcotizing action on mammals as well as on fish (Selye, 1941*a,b,c*; Selye and Heard, 1943). The Dytiscidae have presumably exploited this anesthetic activity of steroids as their main defense against predation, although electrophysiological evidence that steroids affect the spontaneous and evoked neural discharges from the olfactory system of fish (Hara, 1967; Oshima and Gorbman, 1968) has led Clayton (1970) to speculate that the defensive action of steroids may also involve repellency. The pygidial norsesquiterpenes of the Gyrinidae apparently serve a defensive function similar to that of the defensive steroids of the Dytiscidae by also being narcotic to predators.

Since the physiological effects of the norsesquiterpenes are so similar to those of equivalent amounts of the anesthetic steroids, both types of agents may share a similar mode of action. Although steroid anesthesia has been extensively studied (see Kappas and Palmer, 1963 for a review) the mode of action of steroid anesthetics has not been elucidated. However, as characterized in the early reports on the subject (Selye, 1941*a,b,c*; Selye, 1942; Winter, 1941; Selye and Heard, 1943; Selye and Stone, 1944), steroid anesthesia is a nonspecific phenomenon, as numberous steroid compounds of diverse structure are anesthetic. In addition, anesthesia is achieved only when an organism is suddenly flooded by intravenous or intraperitoneal injection of these agents.

Norsesquiterpene narcosis also appears to be a nonspecific phenomenon since gyrinidione, gyrinidal, and gyrinidone represent some diversity of structure, and all are narcotic to fish. Studies of the absorption of norsesquiterpenes and steroids across *P. promelas* gill membranes (Miller and Mumma, 1974) demonstrate that equally rapid flooding is required for norsesquiterpene narcosis as for steroid narcosis.

One distinct difference was noticed between steroid and norsesquiterpene narcosis. Compared with the steroids, the dosage of norsesquiterpene required to be deeply anesthetic to fish lay closer to the dosage that proved lethal. Apparently the therapeutic index of the norsesquiterpenes is lower than that of the steroids.

Although numerous steroids demonstrate excellent anesthetic qualities, the medical use of most of them as anesthetics is barred by their hormonal side effects. Very few steroids have been found to possess both desirable anesthetic qualities and no hormonal activity. One such agent that has been developed and is being used as a basal anesthetic is 5β-pregnan-21-hydroxy-3,20-dione sodium succinate (Clarke et al., 1971). That the highly oxygenated norsesquiterpenes or their analogs may also have value as anesthetics for medical purposes is a possibility that warrants investigation. It seems unlikely that the pharmacological use of norsesquiterpenes would be limited by hormonal activity.

Various oxygenated terpene derivatives that are structurally related to the gyrinid norsesquiterpenes, such as VI–IX, have previously been isolated as defensive agents of arthropods. These compounds have usually been ascribed activity as general toxicants and in some cases insecticidal activity (Pavan, 1952). One compound that is structurally related to the gyrinid norsesquiterpenes and is also wellknown for its neurological activity is nepetalactone (X) the active component of catnip (*Nepeta cataria*) which is excitatory to felids. Whether neurological activity is a widespread attribute of oxygenated terpene derivatives remains to be seen.

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