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PHENOLS AND QUINONES FROM THE DEFENSIVE SECRETIONS OF THE TENEBRIONID BEETLE, *ZOPHOBAS RUGIPES*

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Abstract—The Central American tenebrionid beetle, *Zophobas rugipes*, has a pair of prothoracic defensive glands secreting phenols, and a pair of abdominal defensive glands secreting quinones. The phenol-secreting prothoracic glands open between the head and prothorax and consist of a pair of reservoirs covered with glandular tissue. The orifice is controlled by a muscle, but the milky secretion is presumably expelled by haemolymph pressure and the elasticity of the reservoir. Electron microscopy revealed three cell types: (1) Large cells with large, villus-lined vesicles drained by cuticular ducts, (2) elongated cells through which these ducts pass, and (3) epithelial cells associated with the cuticle of the reservoir. Separation and collection of three free phenols were carried out by gas–liquid chromatography (GLC) on a 15% FFAP column. The predominant component was *m*-cresol (melting point and nuclear magnetic resonance (NMR) of the N-phenylcarbamate). Meta-ethylphenol (u.v., m.p. of N-phenylcarbamate and phenol (u.v., retention time on three columns) were also identified as minor components.

The abdominal quinone-secreting glands are a pair of eversible sacs located between the fifth and sixth visible sternites. Their gross anatomy is similar to the homologous glands in *Tenebrio molitor*. Three quinones (GLC on 5% SE-30) were identified (u.v. NMR) as benzoquinone, toluquinone, and ethylquinone. Unidentified minor components were also present.

INTRODUCTION

AMONG the arthropods, chemical defence substances play an important rôle in the relationship of the organism to its predators. Many groups have evolved chemical defences which are variations on a number of basic themes (EISNER and MEINWALD, 1966). Among the subfamily Tenebrioninae, a wide variety of defensive glands has evolved from a pair of invaginations between the fifth and sixth visible abdominal sternites (ROTH, 1945). In all Tenebrioninae studied to date, these glands secrete a mixture of quinones, frequently in combination with hydrocarbons and carbonyl compounds (SCHILDKNECHT *et al.*, 1964; EISNER and MEINWALD, 1966).

In addition to these abdominal or 'anal' glands, there is sometimes present a pair of glands and reservoirs lying in the prothorax and opening on the membrane of the

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neck. In those species of tenebrionids in which the secretions of these glands have been studied, they have been identical with the secretion of the abdominal glands of that species, i.e. *Tribolium confusum* Duval and *T. castaneum* Herbst (LOCONTI and ROTH, 1953; ENGELHARDT *et al.*, 1965), *T. brevicornis* Lec., *T. destructor* Uytt. (Tschinkel, unpublished), and *Diaperis maculata* Olivier (ROTH and STAY, 1958).

The large Central American tenebrionid, *Zophobas rugipes* Kirsch, exhibited certain unusual features: whereas the abdominal defensive glands secrete quinones as other tenebrionids, the prothoracic defensive glands secrete phenols instead of quinones. The purpose of this study was to describe these defensive glands and their secretions.

METHODS

Analysis of the secretions

A drop of milky-white secretion with a strong phenolic odour appeared between the head and prothorax of *Z. rugipes* adults when these were tapped on the head (Fig. 1). This secretion was taken up in a capillary tube and stored frozen in a vial. The yield of secretion from about 3000 beetles was approx. 3 ml. Males released secretion less frequently than females by this method.

The phenolic secretion from the prothoracic glands was dissolved in carbon disulphide and dried with anhydrous magnesium sulphate. It was then resolved by gas–liquid chromatography (GLC) on a 15% FFAP column (Aeropak-30; $\frac{1}{4}$ in. × 10 ft; 210°C) using a Varian Aerograph Model 700 (thermal conductivity detector) with helium as a carrier gas (60 ml/min). The peaks were collected on glass-wool packed tubes cooled with dry ice. All peaks were subjected to u.v. spectroscopy in methanol and baseshifts were measured in methanol–KOH. If enough material was collected, the N-phenylcarbamate was prepared for melting-point determinations and NMR spectroscopy.

The secretion from the abdominal glands appears to be a single-phase, orange to pale violet in colour, and containing cellular debris. It was collected by wiping the everted glands (Fig. 2) with small pieces of filter paper and extracting the pieces immediately with carbon disulfide. The odour and u.v. spectrum indicated the quinonic nature of this secretion. In analysing for low-boiling minor components, a small amount of secretion was collected directly in a capillary.

The quinonic secretion from the abdominal glands was resolved on a column ($\frac{1}{4}$ in. × 5 ft) of 3% NPGS using a Varian Aerograph Model 1200-1 (hydrogen flame detector) with a 10 : 1 splitter and microcollector (75°C; N_2 carrier gas at 60 ml/min). The peaks were individually collected in dry ice-cooled tubes and used directly for u.v. (solvent: methanol) and nuclear magnetic resonance (NMR) (solvent: carbon disulfide) spectroscopy. NMR spectra were taken on a Varian A-60 Nuclear Magnetic Spectrometer. Ultraviolet spectra were taken on a Cary 14 Recording Spectrophotometer.

Morphology of the glands

Attempts to obtain sections from paraffin embedded glands failed, and it was necessary to fix in veronal acetate-buffered osmium tetroxide and embed in

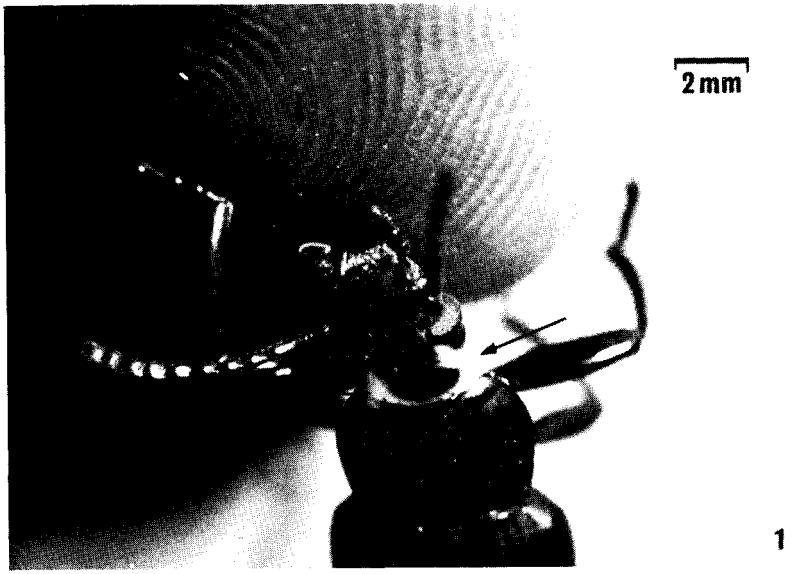

FIG. 1. *Zophobas rugipes* showing two milky droplets of phenolic secretion (arrows) discharged from the prothoracic defensive glands in response to tapping the head.

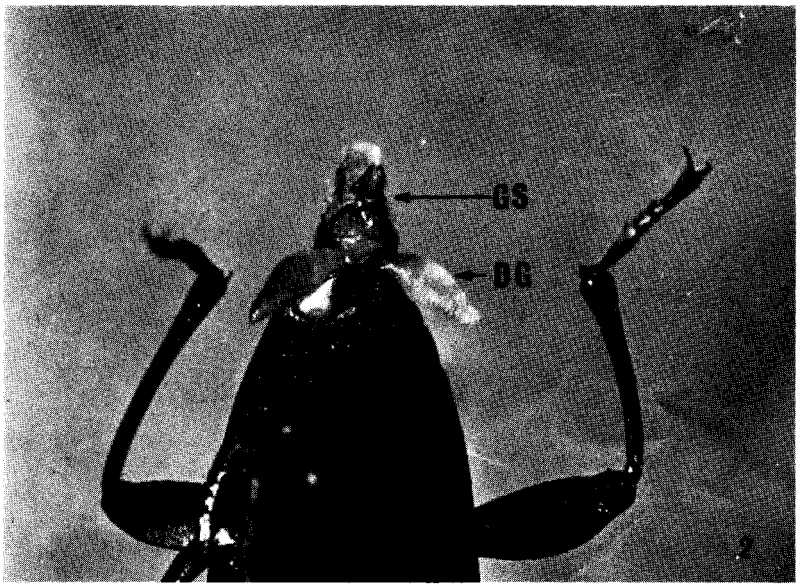

FIG. 2. The everted abdominal defensive glands (DG) of *Zophobas rugipes* in ventral view. Genital segments (GS) are also extruded owing to pressure of the forceps.

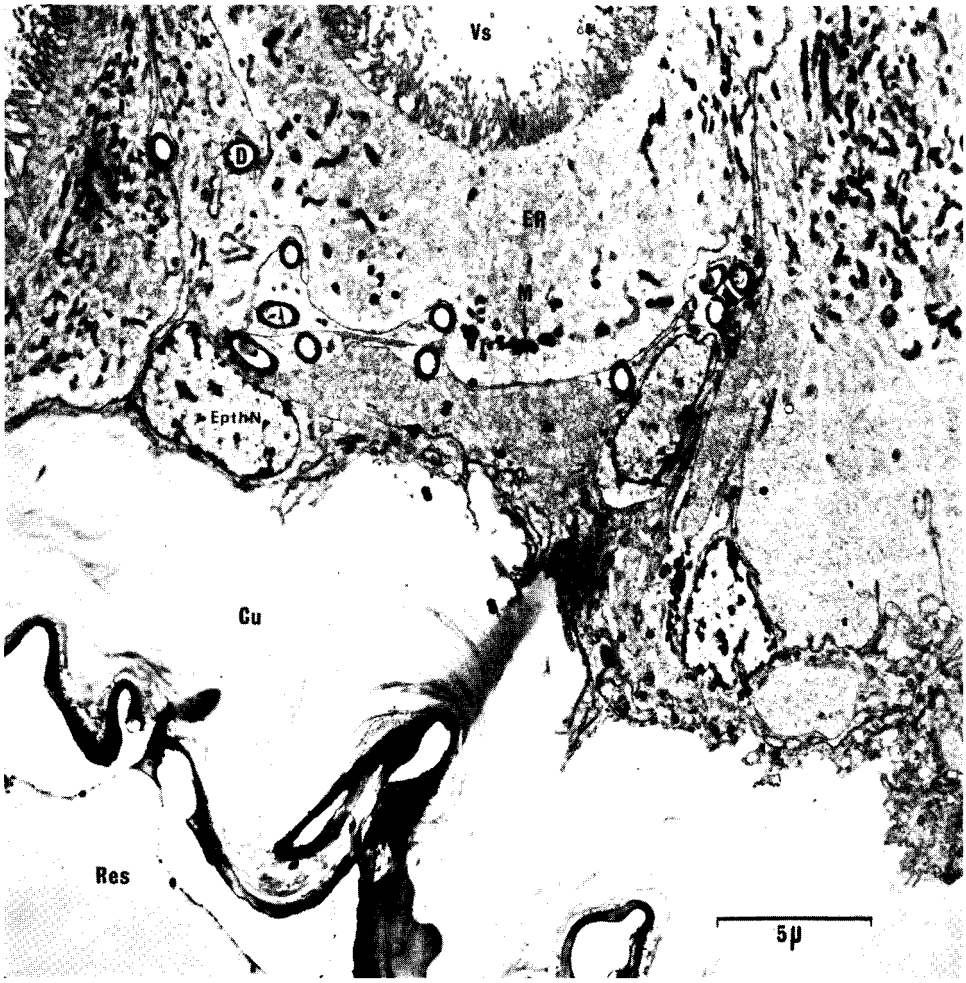

FIG. 4. Low-power electron micrograph of a section through the phenol-secreting epithelium of the prothoracic defensive glands of *Zophobas rugipes*. Vs, vesicle; Vi, villi; Res, reservoir; EpthN, epithelial cell nucleus; Cu, cuticular reservoir sac; M, mitochondria; D, cuticular ducts draining the vesicle; ER, endoplasmic reticulum.

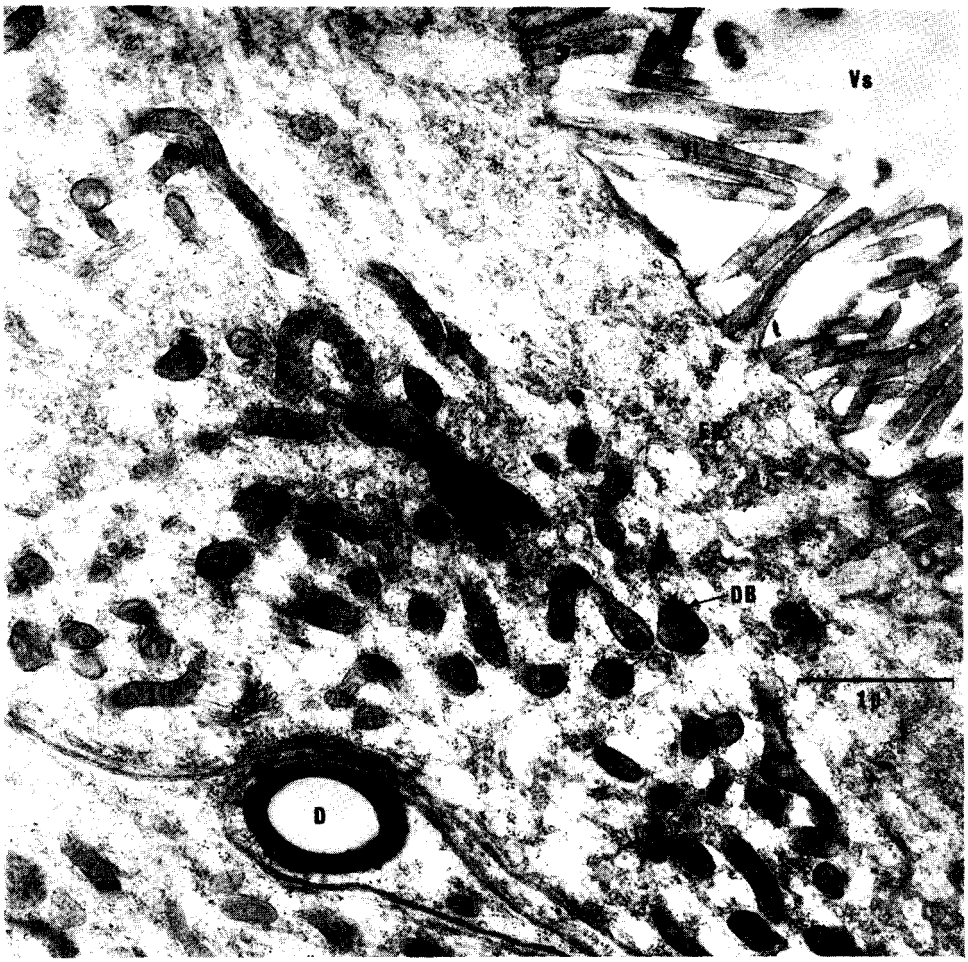

FIG. 5. Higher power electron micrograph of the same section as Fig. 6. Note the tubular reticulum, the mitochondrial dense bodies (DB), and the small Golgi apparatus (G).

Maraglas/DER-732. Sections made with a glass knife on a Porter–Blum Ultramicrotome were stained with toluidine blue for light microscopy and with osmium for electron microscopy.

Life history and rearing

Zophobas rugipes were collected near San Jose, Costa Rica from a man-made cave in which fruit and vampire bats roosted during the day. The adults were collected from the walls of the cave and the surface of the fruitbat guano mass in which the larvae lived. Like many tenebrionids, this species appears to be a general feeder, and in the laboratory they were reared exclusively on wheat bran and water. On this diet the larvae reached maturity in about 4 to 6 months at a weight of 0·6 to 1·0 g. Under crowded conditions, larvae do not pupate and it was necessary to isolate them for a period of 10 to 14 days in order to obtain pupae (Willson and Tschinkel, in preparation). Adults were kept in flat cages with water bottles and screen tops. Under these conditions they lived from 3 to 15 months.

Morphology

Prothoracic defensive glands. The cuticle-lined reservoirs lie in the prothorax and open on the membrane between the prothorax and head near the lateral angles of the former (Fig. 3). The orifice of the gland is formed by a crescent of cuticle

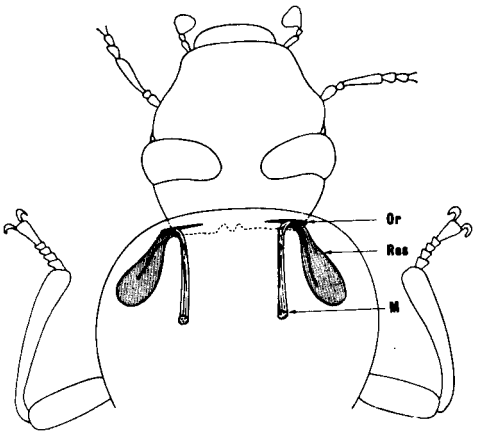

FIG. 3. Schematic drawing of a dissection of *Zophobas rugipes* showing the positions of the prothoracic defensive gland reservoir sac (Res), the valve and orifice (Or), and the valve muscle (M).

normally pressed, by virtue of its elasticity on one side, against the opposite side of the orifice formed by the inside of the lateral prothoracic angle. A muscle, attached near the elastic part of the orifice, opens the valve upon contraction and allows the secretion to be forced out, presumably by haemolymph pressure. The elastic

nature of the reservoir must also play a rôle, since secretion is ejected when the free end of the valve muscle is pulled on a gland which has been dissected free from the body. In very small beetles these structures would be difficult to detect. ROTH (1943) puzzled over the apparent absence of such a valve mechanism in *Tribolium confusum*.

The electron micrographs in Figs. 4 and 5 show the wall of the cuticular gland reservoir convoluted into tight folds which allow great expansion as the sac fills with secretion. The cuticle itself consists of three layers: adjacent to the sac lumen is a thin, very electron-dense layer, next is a somewhat thicker, less electron-dense layer and outside this a very thick fibrous layer of low electron density. The glandular epithelium which surrounds this cuticular sac is essentially one cell layer thick and consists of three major cell types:

(1) Compact epithelial cells which appear to secrete the cuticle lining the reservoir.

(2) Large cells with very numerous small, elongated mitochondria containing electron-dense bodies; a partially granular tubular reticulum; a minimally developed Golgi apparatus; and a large, microvillus-lined vesicle from which a cuticle-lined duct(s), after many convolutions, empties into the reservoir sac (Fig. 4).

(3) Elongate cells carrying the cuticle-lined ducts and possibly responsible for their formation (Fig. 5). The cuticle of the ducts is composed of a thin inner, electron-dense layer surrounded by a thicker, less electron-dense layer. These two layers are probably continuous with the two inner layers of the reservoir sac. In favourable sections these cuticular ducts can be seen to be extracellular products, for they lie outside the plasma membranes of the tubule-carrying cells.

Abdominal defensive glands. The morphology and ultrastructure of the abdominal defensive glands of *Zophobas* were not examined but are probably much like that of *Tenebrio* (LENGERKIN, 1925; ROTH, 1945) in view of the similarity in gross appearance of the glands, defensive behaviour, and quinonic secretion. Using GLC I have found that *Tenebrio molitor* secretes toluquinone, some ethylquinone, and a trace of benzoquinone. SCHILDKNECHT *et al.* (1964), using the less sensitive method of thin-layer chromatography, report only toluquinone in the secretion.

Prothoracic defensive secretion. The secretion of the prothoracic defensive glands is generally a milky emulsion of phenols in phenol-saturated water. In young animals it is often unsaturated and hence clear. The capacity to replenish the secretions appears to decrease with age. The secretion showed three major phenolic peaks which, by co-injection with known phenols, appeared to be phenol, *m*- or *p*-cresol, and *m*- or *p*-ethylphenol in order of their elution from FFAP (Fig. 6). The meta and para isomers of both cresol and ethylphenol could not be resolved on four different liquid phases (SE-30; NPGS; FFAP; QF-1) and tentative identification had to await the determination of melting points of the N-phenylcarbamates. Tables 1 to 3 summarize the data relevant to the identification of peaks I, II, and III from GLC on FFAP. An insufficient amount of

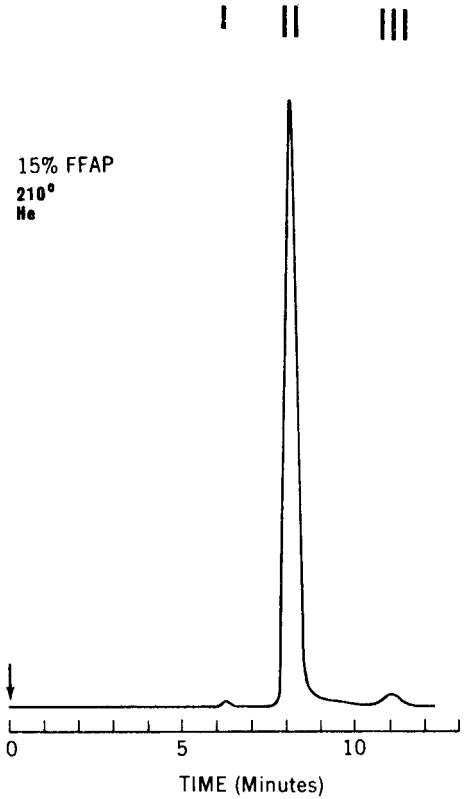

FIG. 6. Gas–liquid chromatography of the phenolic secretion of the prothoracic defensive glands of *Zophobas rugipes* on 15% FFAP (210°C; ¼ in. × 10 ft; He at 60 ml/min; detector 230°C; injector 230°C).

peak I was collected to allow a derivative melting point determination, but by u.v. and co-injection with known compounds on three different liquid phases it was identified as phenol. Peak II was shown to be *m*-cresol by melting point and NMR of the N-phenylcarbamate. Peak III was chromatographed twice on FFAP to achieve purity and was then shown to be *m*-ethylphenol by u.v. of the free phenol and the melting point of the N-phenylcarbamate.

By a comparison of the areas of peaks I, II, and III, the ratio of phenol to meta-cresol to meta-ethylphenol in the secretion was shown to be about 1 : 200 : 4 respectively. Five non-phenolic minor peaks totalling no more than 1 per cent of the quantity of phenols were also present but were not identified.

Abdominal defensive secretion. Gas–liquid chromatography of the secretion of the abdominal defensive glands showed only three peaks which by comparison of retention times appeared to be *p*-benzoquinone, *p*-toluquinone, and *p*-ethylquinone in order of their elution from NPGS (Fig. 7). This was confirmed by u.v. and NMR as shown in Table 4. The low extinction coefficient of peak I indicated a

TABLE 1—IDENTIFICATION OF PEAK I (15% FFAP)

Gas chromatography:
Co-injection of peak I with phenol on:
15% FFAP, 180°C Single peak, 8·7 min
5% SE-30, 75°C Single peak, 2·3 min
3% NPGS, 100°C Single peak, 8·5 min
Ultraviolet spectra: (all wavelengths in mμ)

Solvent	Peak I	Phenol
Methanol	max. 279	max. 278
	273	272
	265 (shoulder)	266 (shoulder)
	min. 241	min. 239
Methanol–KOH	max. 292	max. 291
	236	236
	min. 261	min. 261
	225	225

TABLE 2—IDENTIFICATION OF PEAK II (15% FFAP)

Comparison of the melting points of the N-phenylcarbamates of:

m-Cresol	Peak II	*p*-Cresol
122°C	122·5–123·5°C	115°C
mixed 122·5–123·5°C		mixed 90–110°C

NMR spectrum of peak II-N-phenylcarbamate superimposable over that of *m*-cresol-N-phenylcarbamate.
Singlet: 2·34 ppm
Aromatic—H: 7·02; 7·17; 7·29; 7·39 ppm

(*m*- and *p*-cresol were not separated by gas chromatography)
Peak II yielded 80 mg of the N-phenylcarbamate.

possible impurity. Rechromatography on 3% STAP (70°C, $\frac{1}{8}$ in. × 5 ft, N_2 flow 12 ml/min) resolved a small peak with a retention time of 2 min (benzoquinone: 1 min) and making up about 5 per cent of the mixture. It was not possible to collect enough of this compound for identification. By comparison of peak areas the ratio of benzoquinone to toluquinone to ethylquinone was found to be 1·0 : 1·5 : 1·6.

The apparent absence of a significant component which might act as solvent for the normally solid quinones led to an experiment in which the three quinones were mixed in the ratio in which they are found in the secretion. The melting point of this mixture was sufficiently depressed to form a partially liquid mixture

TABLE 3—IDENTIFICATION OF PEAK III (15% FFAP)

Ultraviolet spectra (all wavelengths in mμ):

Solvent	*p*-Ethyl phenol	Peak III	*m*-Ethyl phenol
Methanol	max. 285	max. 279	max. 278
	278	274	273
	min. 244	min. 241	min. 242
Methanol–KOH	max. 302	max. 302	max. 301
	228 (shoulder)	228 (shoulder)	228 (shoulder)
	min. 263	min. 257	min. 259

Melting points of the N-phenyl carbamates (Peak III yielded 2·9 mg of N-phenyl carbamate):

p-Ethyl phenol	Peak III	*m*-Ethyl phenol
119–119·5°C	140·5–141·5°C	140–141°C
	mixed less than 110°C	mixed 140·3–141·7°C

(*m*- and *p*-ethyl phenol were not separated by gas chromatography.)

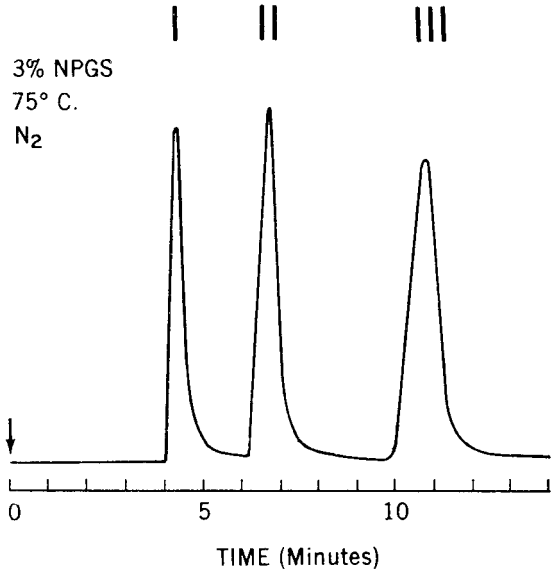

FIG. 7. Gas–liquid chromatography of the quinonic secretion of the abdominal defensive glands of *Zophobas rugipes* on 3% NPGS (75°C; ¼ in. × 5 ft; N_2 at 60 ml/min; detector 120°C; injector 115°C).

at room temperature. Melting was complete at about 40°C. No doubt the substance eluted together with the benzoquinone peak from NPGS lowers the melting point further.

TABLE 4—IDENTIFICATION OF THE QUINONES IN THE ABDOMINAL DEFENSIVE GLAND SECRETION OF *Zophobas rugipes*

Compound	u.v. max (mμ)	Extinction coefficient	NMR (ppm)
Peak I	242	8,100	Singlet 6·98
p-Benzoquinone	242	18,200	Singlet 6·98
Peak II	246	15,000	Singlet 2·08 Aromatic H 6·58; 6·72
p-Toluquinone	246	13,800	Singlet 2·08 Aromatic H 6·58; 6.72
Peak III	247	16,000	Triplet 1·18; J = 0·11 Quartet 2·45; J = 0·11 Aromatic H 6·69; 6·52
p-Ethylquinone	246	—	Triplet 1·18; J = 0·11 Quartet 2·45; J = 0·11 Aromatic H 6·69; 6·25

A search for other minor components which contribute to complete melting was undertaken. Secretion which had been taken up directly into a capillary tube and was thus free of solvent was drawn into a Hamilton syringe and injected onto a 5% SE-30 column at 55°C at low attenuation. Two small peaks were observed at 0·5 and 0·8 min but were not identified. In addition, there were two small broad peaks which eluted only at elevated temperatures. Extraction of the secretion with sodium bicarbonate gave a fraction greatly enriched in the earlier peak. Methylation of the bicarbonate fraction with ethereal diazomethane decreased the retention time of this peak (4·3 min at 90°C on 5% SE-30, N_2 at 28 ml/min), suggesting that it might be an acid. MEINWALD and EISNER (1964) demonstrated octanoic acid in the defensive secretion of the tenebrionid *Eleodes longicollis*, but the retention time of the methylated unknown was intermediate between methyl octanoate and methyl decanoate. The nature of this acidic component is thus not known at present.

When the secretion was treated with sodium hydrosulphite (which reduces quinones to hydroquinones) and extracted with 0·1 N NaOH to remove all acidic compounds, the remaining neutral fraction contained no volatile compounds detectable by GLC even at very low attenuation.

DISCUSSION

The striking feature of the two pairs of defensive glands of *Zophobas rugipes* is that they secrete two different, though related, classes of compounds, phenols and quinones. Phenolic compounds are known to be precursors to various quinones. For example the phenol tyrosine is incorporated into the *p*-benzoquinones of the

defensive secretion of the desert tenebrionid beetle *Eleodes longicollis* (MEINWALD *et al.*, 1966). It is thus reasonable to suppose that in *Zophobas* biosynthesis proceeds through phenolic precursors which are secreted as phenols in the prothoracic defensive glands but are further oxidized to yield quinones in the abdominal defensive glands. Nevertheless, the relative rates of synthesis of the three compounds in each class is different for the two pairs of glands. Thus, whereas the three quinones are present in roughly equal quantities, *m*-cresol is by far the predominant phenol.

Although *Zophobas* is the first reported instance of a phenol-secreting tenebrionid, it is not the first case of a phenolic defensive secretion. The millipede *Abacion magnum* secretes *p*-cresol while the carabid beetle *Chlaenius cordicollis* secretes *m*-cresol (EISNER *et al.*, 1963). Other species of both millipedes and carabids are known to secrete quinones and it is possible that here too the quinone and phenol biosyntheses are related as has been hypothesized for *Zophobas*.

Anatomically, the abdominal defensive glands of *Zophobas* closely resemble those of *Tenebrio molitor* (LENGERKEN, 1925; ROTH, 1945); these probably represent the ancestral form of the more highly specialized glands of such species of tenebrionids as *Eleodes* which do not evert the gland sac but are able to eject the secretion for a distance of 50 cm. Species such as the large woodland *Coelocnemis* and *Cibdelis* may represent intermediate stages, for while their glands are large and well developed, they neither evert the glands nor squirt the secretion but allow it to ooze out over the surface of their bodies. These apparently homologous structures, while almost ubiquitous in the subfamily Tenebrioninae, are absent from the Tentyriinae and Asidinae. Presence of these defensive glands possibly provides a diagnostic character for the Tenebrioninae, allowing this subfamily to be identified by their odour alone.

The systemic distribution of the prothoracic defensive glands is not clear at present. The genera *Diaperis*, *Zophobas*, and *Tribolium* are in the closely related tribes Diaperini, Tenebrionini, and Ulomini, respectively. The glands of all three genera appear superficially similar in structure and position and may well be homologous. Presence of prothoracic defensive glands is the exception rather than the rule in the Tenebrionini, but the frequency of their occurrence in the other two tribes is unknown.

Many ultrastructural similarities between the phenol-secreting glands of *Zophobas* and the quinone-and-hydrocarbon-secreting glands of *Eleodes longicollis* (EISNER *et al.*, 1964) are apparent. Both possess a large, villus-lined vesicle with a cuticular apparatus projecting into it, a tubular endoplasmic reticulum as is frequently found in cells secreting materials other than proteins and numerous small mitochondria with electron-dense inclusions. In both species, the tubules appear to be extra-cellular products outside the plasma membranes of the cells which surround them. The phenol-secreting cells of *Zophobas* differ from cell type 1 of *Eleodes* (EISNER *et al.*, 1964) in that the tubular endoplasmic reticulum does not project into the villi, but are similar in that a secretory unit consists only of the large secretory cell and the tubule-carrying cell(s). The secretory unit of

cell type 2 of *Eleodes* consists of two different but closely associated secretory cells along with the tubule-carrying cell(s). In type 2, the path of the tubule from the cells to the reservoir is fairly direct as opposed to the long convoluted path in type 1 and in the *Zophobas* cells.

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