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QUINONE AND HYDROCARBON PRODUCTION IN THE DEFENSIVE GLANDS OF *ELEODES LONGICOLLIS* AND *TRIBOLIUM CASTANEUM* (COLEOPTERA, TENEBRIONIDAE)

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Abstract—The defensive glands of tenebrionid beetles produce a mixture of benzoquinones and hydrocarbons. These reactive toxicants are manufactured within a complex morphological system, consisting of secretory cells, secretory vesicles within these cells, and cuticular organelles and tubules which carry the products to the gland reservoir. Histochemical and thin-layer chromatographic techniques were employed to follow the process of toxicant production within these cells.

Chromatographic evidence indicated the presence of both *p*-diphenols and their glucosides in extracts of the secretory cells, which suggested that quinones are generated from phenol glucosides by hydrolysis and subsequent oxidation. Histochemical tests demonstrated concentrations of phenolic materials in the cytoplasm of one cell, type 2a, and on the basis of their sensitivity to emulsin, it was concluded that these are phenol glucosides. Glucosidase activity, as shown by a modified bromonaphthol–glucoside technique, was associated with the margins of the vesicle in the same cell type. The cuticular organelle, within this vesicle, yielded positive reactions for a phenolase both with dopa and with N-phenyl-*p*-phenylene diamine as substrates. Along the efferent tubule which runs from this organelle to the gland reservoir, a haemoprotein peroxidase was detected by the benzidine peroxidase test. Each of these steps in quinone production was associated with a particular morphological compartment, and it is apparent that the cytoplasm of the secretory cell is never exposed to the toxic product of the secretory unit.

Lipid reserves, which are possible precursors of the hydrocarbon components in the secretory end-products, were found in all secretory cells. However, the densest deposits were concentrated in the type 1 units, and in these units, a paraoxon-resistant carboxylic esterase was demonstrated with indoxyl substrate, along the margins of and within the secretory vesicle. Deposits of unsaturated lipids were detected in the cuticular organelle of this vesicle.

INTRODUCTION

MANY tenebrionid beetles manufacture and store large quantities of *p*-benzoquinones within their defensive glands (ROTH and EISNER, 1962; SCHILDKNECHT,

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1963; EISNER and MEINWALD, 1966; WEATHERSTON, 1967). The efficiency of these secretions in repelling both vertebrate and invertebrate predators attests to the broad toxicity of the mixtures, and indeed benzoquinones rapidly react with a wide variety of cellular components (SEXTON, 1963). Yet in tenebrionid beetles, and certain other arthropods, quinones are manufactured within the secretory machinery of such glands: how do these cells avoid self-poisoning? It is our intent to approach this problem by attempting to trace the course of toxicant production in two tenebrionids, *Eleodes longicollis* and *Tribolium castaneum*.

In both *E. longicollis* and *T. castaneum*, the defensive fluid is a two-phase system. The non-polar phase contains three *p*-benzoquinones (LOCONTI and ROTH, 1953; CHADHA *et al.*, 1961), one or more unsaturated hydrocarbons, and at least in *E. longicollis*, caprylic acid (MEINWALD and EISNER, 1964). Fig. 1 indicates the

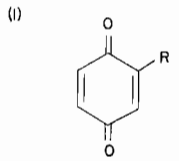


FIG. 1. Components in the defensive secretions of *E. longicollis* and *T. castaneum*. (I) In *E. longicollis* R = -H, $-CH_3$, or $-CH_2CH_3$; in *T. castaneum* R = $-CH_3$, $-CH_2CH_3$, or $-OCH_3$. (II) In *E. longicollis* $n = 5$, 7, or 9. (III) Reported only in *E. longicollis*.

chemical structure of these components. The aqueous phase in *E. longicollis* contains glucose and water (HURST *et al.*, 1964). The presence of glucose in the secretory end-product suggests that the quinones are derived from diphenol glucosides, characteristic precursors of quinones in tanning of structural protein and lipids (BRUNET and KENT, 1955; SEKERIS, 1964). On the basis of histochemical evidence, ROTH and STAY (1958) have indicated that such a pathway is employed for quinone production in the defensive glands of a cockroach, *Diploptera punctata*. Further, SCHILDKNECHT and KRÄMER (1962) have found free diphenols in the defensive secretion of *Tenebrio molitor*, a quinone-producing tenebrionid. In another parallel to cuticular systems, the hydrocarbons in the secretions of *Eleodes* and *Tribolium* may be derived from fatty acids, since fatty acids are presumed to be the likely precursors of analogous terminally unsaturated hydrocarbons in insect epicuticle (GILMOUR, 1965; GILBY, 1965).

The morphology of the secretory machinery is complex, and has been described by ROTH (1943) for *Tribolium confusum*, PALM (1946) for *T. destructor*, and EISNER *et al.* (1964) for *E. longicollis*. The latter study extended the morphological analysis to the electron microscopic level. There are two types of secretory units, each with its own secretory vesicles, cuticular organelles, and efferent tubules. On the basis of the *Eleodes* study, EISNER *et al.* (1964) suggested that these various morphological compartments might be 'reaction chambers' in which the final steps of toxicant production could take place.

MATERIALS AND METHODS

Materials

Tribolium castaneum (Herbst) were obtained from a stock culture of the McGill strain which was raised on a mixture of whole-wheat flour and brewer's yeast. *Eleodes longicollis* Leconte were collected in south-eastern Arizona and maintained in the laboratory on a diet of dog food, pablum, and lettuce.

Thin-layer chromatography

Crude defensive secretion itself and methanolic extracts of the secretory cells were spotted on to silica gel-G chromatoplates. The plates were developed in benzene–dioxane (3 : 1), chloroform–ether (2 : 1), both after SCHILDKNECHT and KRÄMER (1962); or in chloroform–methanol (2 : 1). Phenols were detected by spraying with ferric-ferricyanide or diazotized sulphanilic acid.

Morphological studies

For morphological examinations glands were dissected out of the insects and examined under phase-contrast as fresh squash preparations, or fixed in alcoholic Bouin's, Helly's fluid, or osmium tetroxide (1% in pH 7·4 phosphate buffer for 3 hr). Whole mounts or sections were examined after staining with Delafield's haematoxylin, Mallory's trichrome, or in the case of osmium-fixed material ethyl gallate (WIGGLESWORTH, 1957).

Histochemistry

For oxidases, esterases, and phosphatases, tissues were fixed in cold (0–4°C) formol–calcium or buffered formaldehyde (SABATINI *et al.*, 1963). For the bulk of the histochemical studies, whole mounts were examined, although, as noted, gelatine and cryostat sections were used in certain techniques.

Phenols

The ferric-ferricyanide test (LILLIE, 1954) was utilized for tissues fixed in formaldehyde or ethanol. A positive result for phenols was assumed only when the reaction was unaffected by sulphydryl or amino-blocking agents (PEARSE, 1960). Tissues were freeze-dried or ethanol-fixed prior to tests with ammoniated silver nitrate (PEARSE, 1960), or silver nitrate at lower pH's (LILLIE *et al.*, 1957). Okamoto's 'glucose test' was used after the appropriate freeze-substitution (PEARSE, 1960). As a control procedure, phenols were blocked by a 24 hr pretreatment in diazomethane (ethereal solution 4°C).

Lipids

Some glands were fixed in formol–calcium and flooded with Oil red O in *iso*-propanol (LILLIE, 1954) or treated with Sudan III and IV in acetone–ethanol (PEARSE, 1960, after KAY and WHITEHEAD, 1941) or immersed in Sudan black B in ethylene glycol (CHIFFELLE and PUTT, 1951). Other tissues were chromed (BAKER,

1946) and stained with Sudan black B (GURR, 1958, after BAKER, 1949). For controls, lipids were extracted with methanol–chloroform (1 : 2 at 60°C for 1–12 hr), absolute acetone (25°C for 24 hr), or with pyridine (BAKER, 1946). The Norton test for unsaturated lipids was performed on glands fixed in formol–cadmium (NORTON *et al.*, 1962). Controls included elimination of the bromination step, performic acid oxidation of the double bonds, extraction with chloroform–methanol, and solubilization of the silver bromide.

β-Glucosidases

A modification of the bromonaphthol method (COHEN *et al.*, 1952) was employed on whole mounts and cryostat sections of fresh tissues. The incubation medium consisted of the following: 6-bromo-2-naphthyl-β-D-glucopyranoside (2·5 mg), N,N-dimethylformamide (0·3 ml), pH 5·3 phosphate–citrate buffer (10 ml), distilled water (15 ml), and Red violet LB (20 mg). The components were added in order and the pH adjusted as necessary. Indican (indoxyl-β-D-glucoside), which upon hydrolysis should give rise to indigo, was applied in pH 5·3 phosphate buffer. The nitro BT tetrazolium method for disaccharidases (DAHLQVIST and BRUN, 1962) was adapted by substituting phenol-β-D-glucoside for sucrose.

Oxidases

With the dopa (3,4-dihydroxyphenylalanine) substrate, incubations were for 12 to 15 hr (BECKER *et al.*, 1935). For the BURSTONE (1961b) oxidase medium, the substrates of choice were N-phenyl-*p*-phenylene diamine and 8-amino-1,2,3,4-tetrahydroquinoline and the incubations ranged from 20 to 40 min. In the benzidine peroxidase test (PEARSE, 1960, after VAN DUIJN, 1955) tissues were incubated for 5 to 10 min. Oxidase inhibitors were: phenylthiourea (10^{-2} M), sodium diethyldithiocarbamate (10^{-2} M), sodium azide, and sodium cyanide. Tissues were soaked in the inhibitors for 1 hr prior to incubation.

Esterases

For the α-naphthyl acetate technique (PEARSE, 1960), incubation was at pH 7·4 for 6 to 15 min and the freed naphthol was coupled with diazonium salts (Blue B, Red TRN, Red violet LB or Garnet GBC). In the naphthyl AS-D acetate technique (BURSTONE, 1957) incubations were at pH 7·1 for 30 min; liberated naphthol was simultaneously captured with Blue RR. When employing 4-chloro-5-bromo-indoxyl acetate as substrate (HOLT, 1958) incubations were at pH's 8·5, 8·1, or 7·1 for 1 to 4 hr. Oxidation catalysts were copper sulphate or equimolar potassium ferricyanide–potassium ferrocyanide. Paraoxon (10^{-3} M) and silver nitrate (10^{-2} M) were used as esterase inhibitors.

Phosphatases

Metal salt procedures were employed for acid phosphatases (HOLT, 1959), alkaline phosphatases (GURR, 1958, after Gomori), and ATPase (WACHSTEIN and MEISEL, 1957; PADYKULA and HERMAN, 1955). Naphthyl-AS substrates for alkaline

phosphatases were according to BURSTONE (1961a) and for acid phosphatases according to BARKA and ANDERSON (1962). Sodium fluoride (10^{-2} M), cysteine (2×10^{-3} M), and *p*-chloromercuribenzoate (2×10^{-3} M) were employed as inhibitors.

THE SECRETORY CELLS: STRUCTURE AND GENERAL HISTOCHEMISTRY

Our morphological data were essentially in agreement with the earlier studies cited above. Although there are slight differences in detail between the secretory cells of *E. longicollis* and *T. castaneum*, the overall structural pattern is similar. A diagrammatic summary of the cell types is provided in Fig. 2. There are two types

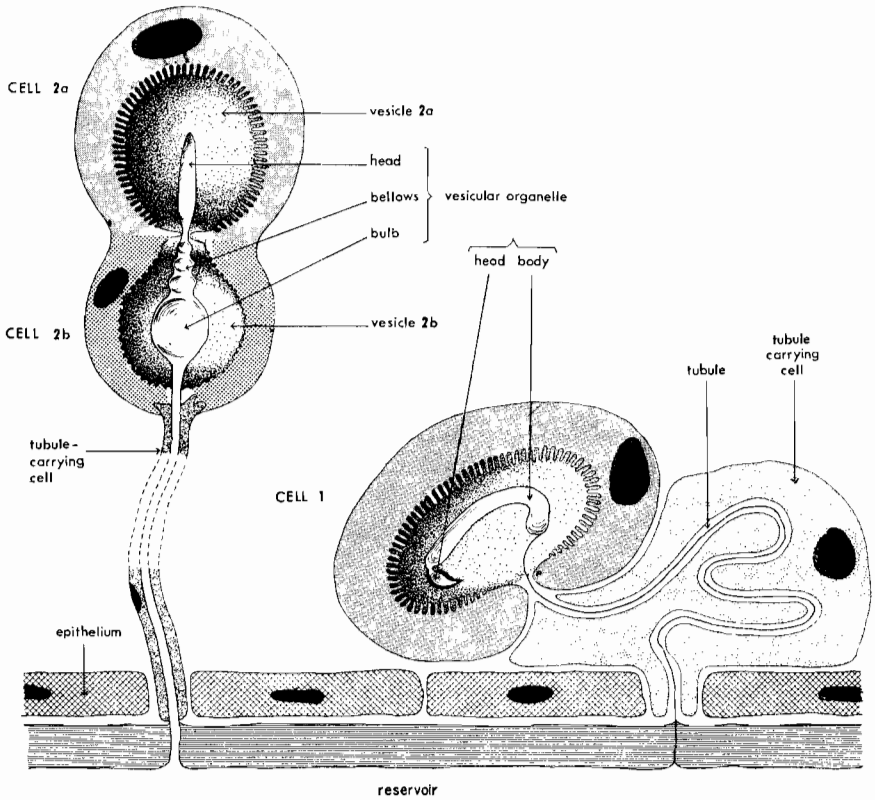


FIG. 2. Reference diagram of the secretory cells in the defensive glands of *E. longicollis* and *T. castaneum*. See text for details.

of secretory units. Type 1 units are single cells which lie on the surface of the reservoir: within each cell is a large secretory vesicle which contains a cuticular organelle. This organelle is divisible into an apical 'head' and a longer cylindrical 'body'. An efferent cuticular tubule runs from the body of the organelle to the reservoir of the gland. The type 2 secretory units consist of two secretory cells;

each cell has a vesicle and each pair of cells share a cuticular organelle. In the vesicle of the more apical cell (2a) lies the 'head' of the organelle, and within the vesicle of cell 2b lie the pleated 'bellows' and dilated 'bulb'. An efferent tubule runs from the bulb to the reservoir.

General tests for carbohydrates and proteins (PEARSE, 1960) yielded only the expected results and will not be further reported here. Phosphatase activity is often associated with tissue sites at which active transport occurs, and therefore was of some interest in this secretory system.

At first, tests for alkaline phosphatase with the Gomori technique yielded a strong reaction, but we later concluded that this was a false positive. The tissues had been fixed in phosphate-buffered aldehyde, washed in running water, and incubated for 20 min in the Gomori medium. The cobalt sulphide product was neatly localized in the vesicle of cell 2a (Fig. 4). Following fixation in phosphate-free aldehydes, however, the Gomori reaction was negative, and the alkaline phosphatase reaction was always negative with naphthyl substrates. If tissues were soaked in solutions containing phosphate at any point prior to the formation of the cobalt salt, the reaction product was present in the vesicles. Apparently phosphate ions are trapped within this vesicle.

In fresh tissues, the tests for ATPase were positive after 1 hr at pH 9·4 or after 4 hr at pH 7·2 at only one site, namely the margins of the vesicles of cell 2a (Fig. 5). The enzyme was inhibited by PCMB but not by cysteine.

With either Gomori techniques or the naphthol-AS method, acid phosphatase activity was found in all cell types (Figs. 6, 7). This reaction was prevented by pretreatment with NaF, and the sites of the activity are similar in distribution to the lysosome-like dense bodies described in *Eleodes* by EISNER *et al.* (1964).

QUINONE PRODUCTION

Phenolic intermediates

Chemical evidence. By thin-layer chromatography, phenolic substances were detected in the defensive secretion itself and in methanolic extracts of the secretory cells of *E. longicollis*. The phenols fell into two distinct classes.

The first group, Class I, moved rapidly in all solvent systems (R_f: 0·7 to 0·95) and was present in both the secretion and the cellular extract. The migration rate on chromatoplates corresponded exactly to that of a mixture of authentic hydroquinone, methyl-hydroquinone, and ethyl-hydroquinone.

The Class II phenols were separated from the origin only by the most polar solvent system (chloroform–methanol), and could be demonstrated only in the methanolic cellular extract. Their rate of movement on the chromatoplates was similar to that of arbutin, the β-glucoside of hydroquinone. Class II material was isolated by preparative thin-layer chromatography, and a portion of this isolated material was exposed to emulsin, a β-glucosidase (0·5% in pH 5·8 phosphate-citrate buffer for 30 min at 28°C). The results are shown in Fig. 3. On the basis of this chromatographic evidence, it appears that the glucosidase converted the

major portion of the Class II phenols to hydroquinones, and thus it is most probable that the Class II substances are β-glucosides of the diphenols in Class I.

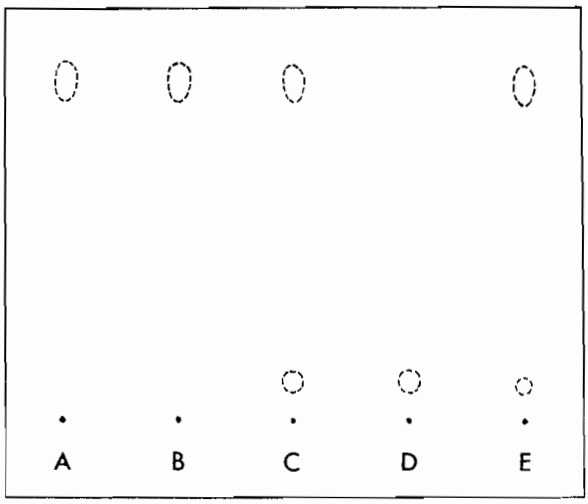


FIG. 3. Drawing of thin-layer chromatogram of phenolic substances in the defensive glands of *E. longicollis*. (A) Crude secretion; (B) mixture of hydroquinone, methyl-hydroquinone, and ethyl-hydroquinone; (C) methanolic extract of the secretory cells; (D) authentic arbutin; (E) lower spot from (C) treated with emulsin and the digest chromatographed. (Silica gel-G, chloroform–methanol.)

Histochemistry. Although reducing substances were demonstrated in both types of secretory units, the strongest reactions were found in the cytoplasm, vesicular organelle, and efferent tubule of unit 2. In the cytoplasm of cell 2a, ammoniated silver nitrate caused darkening in 30 min (Figs. 8, 9, 12); acidic silver nitrate, in 12 to 15 hr; and endogenous cytoplasmic components coupled readily with diazonium salts such as Fast red TRN (Fig. 11). Reduction of ferric ions to produce Prussian blue in the ferric–ferricyanide test (Fig. 10) was rapid in the cytoplasm of this cell; this reaction was blocked by previous exposure of the tissues to diazomethane but not affected by nitrous acid oxidation or sulphydryl blocking reagents. Pretreatment of the fixed secretory cells with emulsin markedly reduced the intensity of the silver reduction, which implicated phenol glucosides as the reactive material at these sites.

In fresh, freeze-dried, or freeze-substituted tissues, silver reduction was marked in the vesicle of 2a, the cuticular organelle of the unit, and the efferent tubule. Reducing material was not present at these sites following aqueous and alcoholic fixation, suggesting that it was readily leached out of the tissues by the fixatives. The Okamoto test (freeze-substitution followed by alcoholic silver nitrate) yielded discrete silver deposits within the cuticular organelle and efferent tubule of the type 2 unit (Fig. 13). When freeze-dried tissues were removed from the desiccating

chamber, or when fresh-frozen sections were obtained, and either plunged directly into ammoniated silver nitrate, darkening occurred in the same organelle and tubule (Figs. 14, 15). Brief hydration (30 sec) prior to this silver nitrate treatment caused material to leak from the organelle into the surrounding vesicle. Acidic silver nitrate produced no darkening in parallel tests. Silver reductions occur in the presence of diphenol glucosides (e.g. arbutin), diphenols, quinones, or glucose. Authentic samples of each were spotted on pieces of filter-paper which were subsequently fixed, freeze-dried, or freeze-substituted, and then tested in the silver solutions. All gave positive reactions with the Okamoto test and with ammoniated silver nitrate; all were leached by aqueous or alcoholic fixatives. However, both free diphenols and arbutin reduced acidic silver nitrate, whereas the substances in the organelle and tubule did not.

Glucosidases

The secretory cells of the tenebrionid defensive glands contain a β-glucosidase. Of the various tests for glucosidases, only the bromonaphthol technique yielded usable histochemical results. Diffusion artifacts were common in longer incubations, but with short incubations activity was highest in the type 2 secretory unit. With fresh-frozen sections, the activity in these cells was concentrated around the margins of the secretory vesicle of cell 2a (Fig. 16).

Oxidases

The enzymes which oxidize diphenols to quinones in these glands are at two sites: the vesicular organelle of secretory unit 2, and the efferent tubules from both units.

FIG. 4. Gomori test for alkaline phosphatase, positive only in the vesicle (vs) of cell 2a; the reaction is a false positive, as explained in the text. (*T. castaneum*, whole mount.)

FIG. 5. ATPase which is weakly positive around the vesicle (vs) of cell 2a. (*E. longicollis*, whole mount.)

FIG. 6. Acid phosphatase in cells 2a+b. (*E. longicollis*, naphthol AS-MX coupled with hexazonium pararosalin, whole mount.)

FIG. 7. Acid phosphatase in cell 1. (*T. castaneum*, Gomori technique, whole mount.)

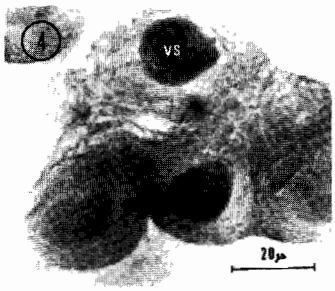
FIG. 8. Silver-reducing substances in cells 2a+b. The reaction is positive in the cytoplasm of cell 2a. (*E. longicollis*, ammoniated silver nitrate, paraffin section.)

FIG. 9. Silver-reducing substances in cells 2a+b. In this unfixed material, the reaction is positive in both the cytoplasm and vesicle (vs) of cell 2a. (*E. longicollis*, ammoniated silver nitrate, unfixed tissue, cryostat section.)

FIG. 10. Ferric–ferricyanide reaction for reducing substances. Only deposits in the cytoplasm of cell 2a are positive. (*T. castaneum*, whole mount.)

FIG. 11. Diazonium coupling for phenols in cells 2a+b. (*T. castaneum*, whole mount.)

FIG. 12. Silver-reducing substances in cells 2a+b. (*T. castaneum*, whole mount.)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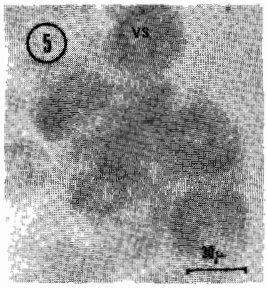
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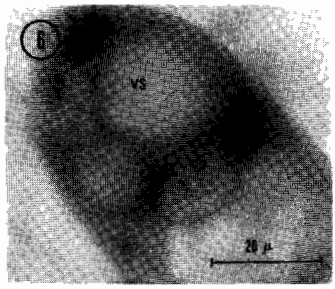
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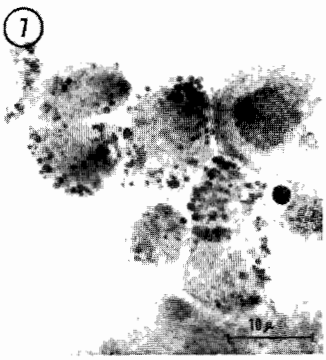
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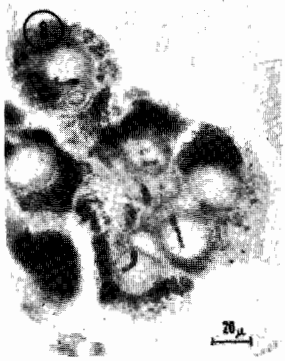
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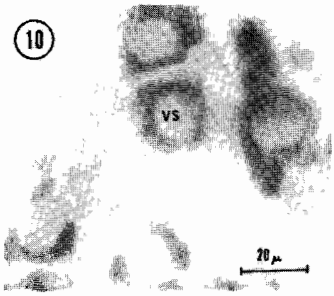
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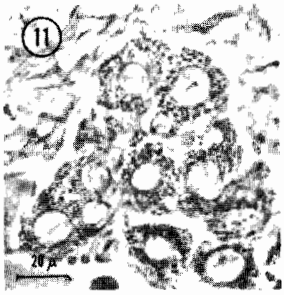
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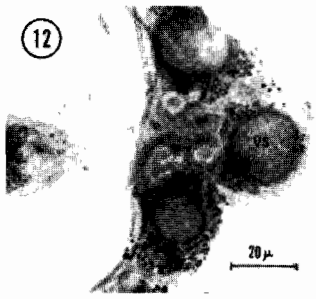
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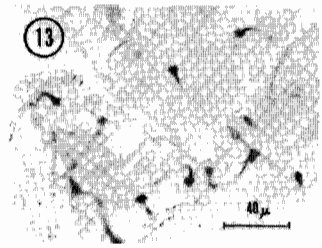
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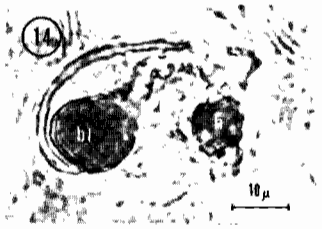
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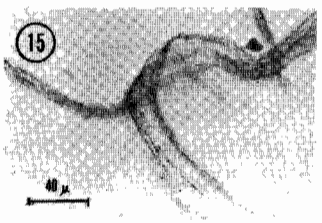
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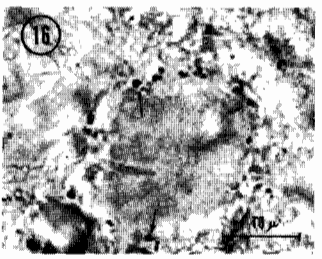
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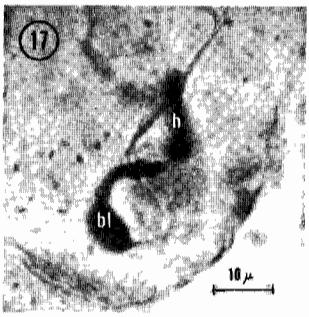
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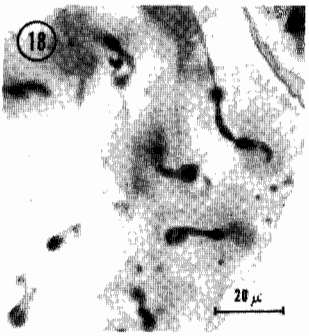
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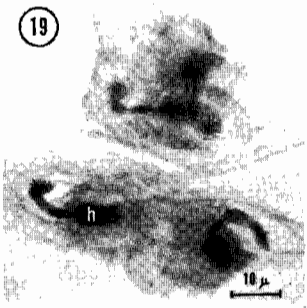
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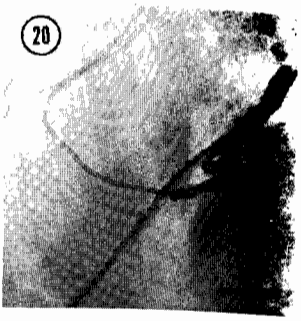
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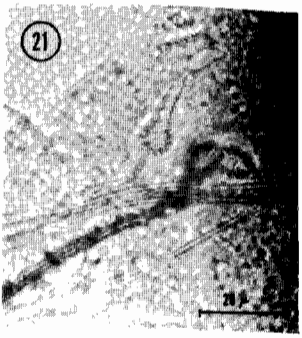
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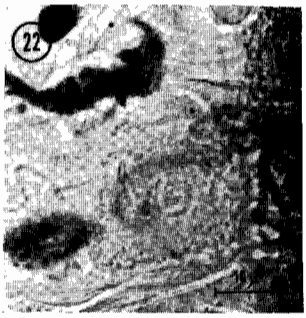
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The oxidase associated with the vesicular organelle was demonstrated with dopa (Figs. 17, 18) or the Burstone reagent (Fig. 19). Its activity was inhibited by phenylthiourea, diethyldithiocarbamate, sodium azide, and sodium cyanide (Table 1); these results suggest that this enzyme is a copper-containing phenolase.

The oxidase found along the efferent tubules was detected only with the benzidine peroxidase test (Figs. 20, 21). On the basis of its requirement for hydrogen peroxide, its sensitivity to azide and cyanide, and its insensitivity to phenylthiourea and diethyldithiocarbamate (Table 1), the enzyme along the efferent tubule from the type 2 units was classified as a haemoprotein peroxidase. Additional peroxidase activity was detected along the efferent tubule from type 1 units (Fig. 22). The resistance of this reaction to ethanol and inhibitors is noted in Table 1.

A coherent picture of the mechanisms employed for quinone production in tenebrionid defensive glands emerges from the chemical and histochemical results. The overall pathway is most probably from phenol glucoside to free diphenol, and then to quinone. Each reaction step appears to be associated with a particular morphological compartment. Among the reducing substances in the cytoplasm of cell 2a are phenolic materials: only phenols and amines should react with diazonium salts and be blocked by diazomethane, and pretreatment with nitrous acid to remove amino groups did not affect the intensity of the reaction. These phenolic

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FIG. 13. Okamoto test for reducing substances. After freeze-substitution, the reducing material was found in the efferent tubule and vesicular organelle of secretory unit 2. (*T. castaneum*, whole mount.)

FIG. 14. Silver-reducing substances in the head (h) and bulb (bl) of secretory unit 2 in unfixed tissue. (*E. longicollis*, fresh tissue, cryostat section.)

FIG. 15. Silver-reducing substances in efferent tubules from secretory unit 2. (*E. longicollis*, freeze-dried, whole mount.)

FIG. 16. β-Glucosidase in the cells of secretory unit 2 as demonstrated by the bromonaphthol glucoside technique. The particles of the azo-coupling product (arrows) tend to be distributed around the margins of the vesicle of cell 2a. (*E. longicollis*, cryostat section.)

FIG. 17. Dopa test for phenol oxidase. The melanic product is found in the head (h), bellows, and bulb (bl) of the vesicular organelle of secretory unit 2. (*E. longicollis*, whole mount.)

FIG. 18. Dopa test for phenol oxidase in secretory unit 2. (*T. castaneum*, whole mount.)

FIG. 19. Burstone test for oxidase. As in Fig. 18, the reaction product is concentrated in the vesicular organelle of secretory unit 2. (*T. castaneum*, whole mount.)

FIG. 20. Benzidine peroxidase test. The reaction is positive along the efferent tubule from secretory unit 2. (*E. longicollis*, whole mount.)

FIG. 21. Benzidine peroxidase test. The positive reaction does not extend into the vesicle of cell 2b. (*T. castaneum*, whole mount.)

FIG. 22. Benzidine peroxidase test. The efferent tubule from type 1 units is positive. (*T. castaneum*, whole mount.)

substances are resistant to aqueous and alcoholic fixatives, and such resistance is more characteristic of glucosides than of free diphenols. The reduction of acidic silver nitrate was sluggish at this site, while the reaction of free diphenols with acidic silver nitrate is rapid (LILLIE *et al.*, 1957). Finally, the sensitivity of these substances to emulsin strongly argues for their identification as diphenol glucosides.

TABLE 1—OXIDASES IN THE GLANDS OF *Tribolium*

	Phenolase (unit 2)		Peroxidase	
	Dopa	Burstone	Unit 2	Unit 1
No inhibitor	++	++	++	++
No substrate	0	0	0	0
Phenylthiourea	0	0	++	++
Sodium diethyl-dithiocarbamate	0	0	++	++
0·005 M KCN	0	0	0	++
0·01 M KCN	0	0	0	0
0·005 M NaN_3	+	+	++	++
0·01 M NaN_3	0	0	0	++
60°C (1 hr)	0	0	++	++
100°C (10 min)	0	0	0	0
100% ethanol	0	0	0	++

0, No activity; +, slight activity; ++, high activity.

Before oxidation of diphenols to quinones, the glucoside must be hydrolysed. Although technical limitations prevented the precise localization of the glucosidase, results with bromonaphthol substrate suggested that some activity was associated with the margins of the vesicle of cell 2a. Certainly this hydrolysis must occur before the phenol oxidase within the vesicular organelle can act, and the presence of glucose in the secretory end-product strongly suggests that the glucosidase acts outside of the cytoplasm of the secretory cells.

In the vesicular organelle, the free diphenols are oxidized to quinones. The dopa and Burstone results, with their inhibitor controls, clearly demonstrate the presence of a copper-containing oxidase. Both ammoniated and alcoholic silver nitrate are reduced, and this reaction may be due to the diphenols, the quinones, or their molecular complex, quinhydrone.

Along the efferent tubule from the type 2 secretory unit is yet another oxidative enzyme, a haemoprotein peroxidase. At first glance, this enzyme appears to be redundant: why should there be a second oxidative enzyme? We believe that the explanation probably lies in the kinetics of the oxidation itself. *p*-Quinone and *p*-diphenols rapidly form a molecular complex, quinhydrone, which is resistant to the phenolase oxidation. Futhermore, the cells are not unusually well tracheated, and thus oxygen may well be a limiting factor within the vesicular organelle. Along

the elongate efferent tubule, however, the oxidation can continue. The presence of free diphenols in the reservoir indicates that this oxidation reaction never goes to completion. Peroxidases in cuticular structures are not uncommon. YAMAFUJI (1934) indicates that quinone tanning of cuticle is partially mediated by a peroxidase, and COLES (1966) has suggested that the cross-linking of resilin chains in elastic cuticle is accomplished by a peroxidase.

The various histochemical tests show clearly that type 2 secretory units contain both the substrates and the necessary enzymatic catalysts for quinone production. A hypothetical plan of quinone production in this unit is shown in Fig. 23. In contrast, only a glucosidase and perhaps a peroxidase were found in the type 1 units. The glucosidase activity was weak and may stem from the lysosome-like bodies described by EISNER *et al.* (1964) in these cells.

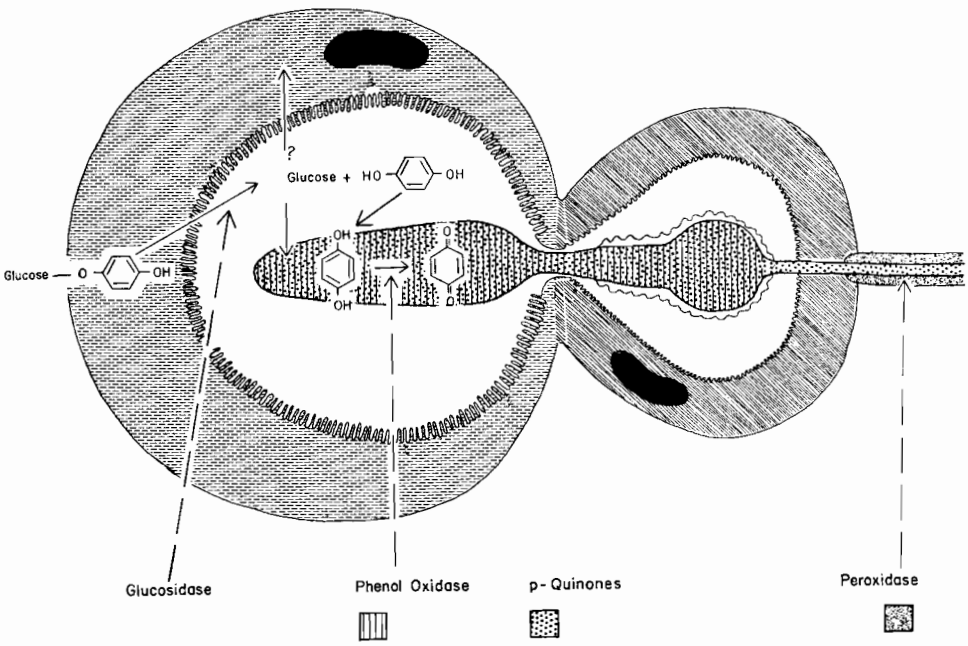

FIG. 23. Hypothetical plan of quinone production in secretory unit 2 of the defensive glands of *E. longicollis* and *T. castaneum*.

HYDROCARBON PRODUCTION

Lipids

Lipid substances are found throughout both secretory units, but they are particularly abundant in secretory unit 1. A variety of solubility techniques reveal deposits of acetone-soluble lipids in the cytoplasm and even in the vesicle of the type 1 cells (Figs. 24, 25, 27).

The acid haematein technique is also positive at these sites. The Norton test, which is based upon bromination of double bonds and subsequent silver reduction, yielded black deposits at only one location—within the vesicular organelle of the type 1 units (Fig. 26). Of the components in the defensive secretion, only the hydrocarbons are positive with this test.

Rather sparse lipid deposits were scattered over the cytoplasm in secretory unit 2, and on occasion distinct lipid deposits could be detected in the vesicles of this unit.

Carboxylic esterases

With both naphthol and indoxyl techniques, cytoplasmic esterases were demonstrated in all secretory cell types (Figs. 28, 30, 31). An additional esterase, distinguished by its resistance to paraoxon, was associated with the vesicle and organelle in unit 1 (Figs. 29, 32).

No direct chemical evidence is available on the pathways employed for hydrocarbon production in the tenebrionid defensive glands. However, the histochemical evidence is entirely consistent with the suggested route: glycerides to fatty acids, to hydrocarbons. Deposits of lipids, presumably fatty acids, are present in the cytoplasm of cell 1, and the esterases are present at the margins of the vesicles and organelle. In their electron microscopic study EISNER *et al.* (1964) found the contents of the vesicular organelles and tubules of type 1 cells to be strongly osmiophilic. Undecene and tridecene darken in the osmium fixative; *p*-quinones do not. Both the Norton test and the osmium reaction indicate that

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FIG. 24. Oil red O for neutral lipids. Deposits are found within the cytoplasm of the type 1 secretory unit. (*T. castaneum*, whole mount.)

FIG. 25. Sudan III and IV in acetone–ethanol, showing particulate lipid deposits in cell type 1. (*T. castaneum*, whole mount.)

FIG. 26. Norton test for unsaturated lipids. A dense silver deposit is found in the vesicular organelle of type 1 units. (*T. castaneum*, whole mount.)

FIG. 27. Sudan black B. The cytoplasm of type 1 cells is strongly stained. (*E. longicollis*, whole mount.)

FIG. 28. Indoxyl acetate technique for carboxylic esterase. Although esterases are present in both secretory units, the reaction in unit 1 is stronger. (*T. castaneum*, whole mount.)

FIG. 29. Indoxyl acetate technique for cytoplasmic esterase, paraoxon inhibition. Only the vesicles of type 1 units are positive; secretory unit 2 shows no activity. (*T. castaneum*, whole mount.)

FIG. 30. Naphthol AS-D technique for carboxylic esterases in secretory unit 2. (*T. castaneum*, whole mount.)

FIG. 31. Indoxyl acetate technique for carboxylic esterases in secretory unit 1. (*E. longicollis*, whole mount.)

FIG. 32. Indoxyl acetate technique for carboxylic esterases. After paraoxon inhibition the reaction is still positive in the vesicle of secretory unit 1. (*T. castaneum*, whole mount.)

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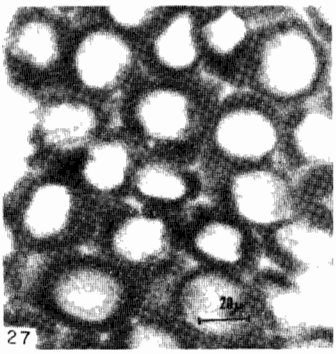

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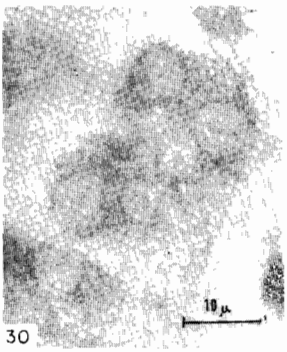

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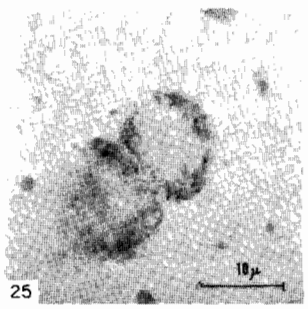

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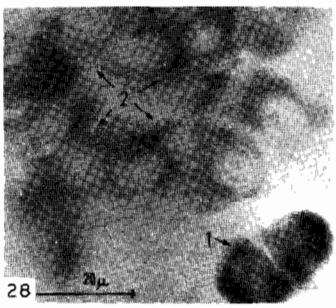

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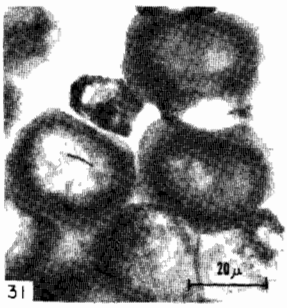

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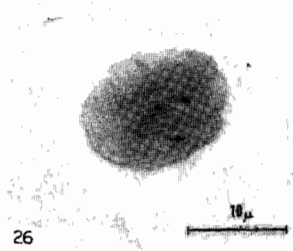

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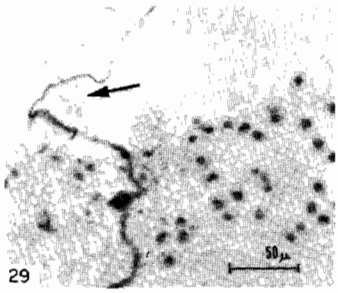

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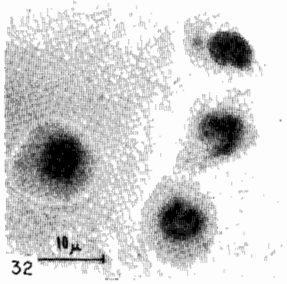

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unsaturated lipids are found within this organelle and the tubule. Although the decarboxylation step could not be demonstrated with appropriate histochemical tests, we presume it occurs between the margins of the vesicle and the efferent tubule.

DISCUSSION

In at least two other defensive glands, those of a polydesmoid millipede and those of a carabid beetle, extracellular chambers are used in the final step of toxicant production. The millipede, *Apheloria corrugata*, emits a mixture of benzaldehyde and hydrogen cyanide from its defensive glands. The final step in the production of these two toxicants is the hydrolysis of a precursor, benzaldehyde cyanohydrin, and this hydrolysis takes place in a special outer chamber of the cuticular reservoir (EISNER *et al.*, 1963). The carabid, *Brachynus crepitans*, ejects a hot mixture of *p*-benzoquinones to repel predators. The final oxidation step occurs in the proximal chamber of the reservoir of its defensive gland. A mixture of diphenols and hydrogen peroxide is passed from distal to proximal chamber, the peroxide is attacked by catalase, and the liberated oxygen converts diphenols to quinones in an exergonic explosive reaction (SCHILDKNECHT and HOLOUBEK, 1961).

In the glands of *Eleodes* and *Tribolium*, the final step in toxicant production also takes place in extracellular compartments, but these compartments are the vesicles and organelles of each secretory unit. The strategy for toxicant production is similar in all three arthropods: only the scale of the reaction chamber differs.

Stepwise segregation of reactions for toxicant production requires three essential adaptations: (1) the compartments themselves, (2) incorporation of the appropriate catalysts into these compartments, and (3) the directed flow of reaction products from one compartment to the next. All three features are found in other insect systems, and their exploitation in these (and presumably many more) defensive glands, hardly requires any dramatic new specializations.

The morphological requirement, the presence of a secretory vesicle, is most easily met. Not only in defensive glands (see EISNER and MEINWALD, 1966 for references) but also in other insect secretory systems, such as the colleterial glands of the cockroach *Periplaneta americana* (MERCER and BRUNET, 1959) secretory vesicles drained by cuticular ductules are found.

For these compartments to serve as reaction chambers, the appropriate catalysts must be present, and in the case of the vesicular organelles, the enzymes must persist in an extracellular structure. We presume that the phenolase of the type 2 secretory unit and the esterase of the type 1 unit are built into the cuticle of the respective organelles; both phenolases and esterases have been reported in other cuticular locations (LOCKE, 1961; LAI-FOOK, 1966; HACKMAN and GOLDBERG, 1967). In the tenebrionid glands, where production of quinones and hydrocarbons continues throughout the life of the beetles, one might suppose that these enzymes would be progressively denatured as the beetles age. This would certainly be true of the phenolase which is inevitably exposed to its quinoid product. For the toxicant production to be continuous, the enzymes must repeatedly be renewed.

It seems likely that the enzymes (at least the phenolase) are derived from pro-enzymes, manufactured within the secretory cells, passed across the vesicular space in the inactive state, and then activated within the organelles. LAI-FOOK (1966) has indicated that such a mechanism is employed to renew the phenolases in the epicuticle of the larvae of *Calpodes*. The inactive proenzyme is present in both the epidermal cells and endocuticle, but the prophenolase is activated only upon abrasion of the epicuticle itself.

If each step in toxicant synthesis is confined to a particular compartment, then the compartments must be arranged so as to channel the flow of the reactants. The linear sequence of compartments in itself would favour an ordered flow of reactants. For reasonable efficiency, back-flow must be minimized. At least for the cuticular components, namely the organelles, EISNER *et al.* (1964) have described morphological features which could be adaptations to this end. The heads of the organelles of both units are covered with perforations, and the organelle of the type 1 units is surrounded by osmiophilic filaments which actually penetrate the wall of this structure. Somewhat similar filamentous structures have been described by LOCKE (1961) in the wax glands of *Calpodes*, and he has further suggested that the precise chemical organization of such filaments could explain the asymmetric permeability properties of certain cuticles (LOCKE, 1965). Not only the cuticular organelles, but also the margins of the secretory vesicles may have special permeability properties. The false positive for the alkaline phosphatase in the vesicle of cell 2a can be interpreted as an indication of facilitated transport of phosphate. Although phosphate readily passes into this vesicle, it cannot easily be washed out.

The most efficient defensive secretions penetrate through cuticular barriers (EISNER *et al.*, 1961; REMOLD, 1962) and yet many are stored for indefinite periods within a cuticular sac, the reservoir of the gland. In certain Heteroptera, REMOLD (1962) has demonstrated that the cuticle of the reservoirs of defensive glands, unlike the body cuticle of the insects, is impermeable to the toxic products stored within it.

Defensive glands are integumentary derivatives. The primary solution to the problems inherent in the manufacture of small, reactive, toxic molecules has been the exploitation of extracellular compartments as reaction chambers for the final steps in toxicant biosynthesis. The preadaptations which allow this strategy to be effective are already present in that complex and versatile organ, the insect integument.

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