

The Terpene-producing Glands of a Phasmid Insect

CELL MORPHOLOGY AND HISTOCHEMISTRY

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ABSTRACT The defensive glands of *Anisomorpha buprestoides* produce the terpene toxicant anisomorpal. Each gland consists of a cuticular secretion reservoir surrounded by the secretory epithelium and the musculature which serves to compress the gland and expel the secretion. Two types of cells make up the secretory epithelium: a squamous layer next to the cuticular reservoir and a layer of larger secretory cells responsible for production of the toxicant. The microvilli-laden plasma membrane of each secretory cell is invaginated to form a central cavity. It appears that secretory products pass into the central cavity and then flow out to the gland reservoir via an efferent cuticular ductule contained within the squamous epithelial cell.

Histochemical techniques demonstrate lipid reserves, carboxylic esterases, a variety of phosphatases, and an alcohol dehydrogenase, within the secretory cells. It is suggested that the lipid reserves are precursors of the terpenoid toxicant, that a mevalonic kinase has been histochemically demonstrated by the phosphatase test, and that an unusual alcohol dehydrogenase is active in the final steps of toxicant synthesis. The histochemical evidence is consistent with the hypothesis that anisomorpal is produced via the mevalonic acid pathway.

Recent studies employing C¹⁴-labeled precursors (Happ and Meinwald, '65; Meinwald, Happ, Labows and Eisner, '66) have demonstrated that at least two insect species possess the biosynthetic machinery for monoterpene production. One such example is the large walking stick, *Anisomorpha buprestoides* (Stoll). When attacked by a predator, this phasmid repels the attacker with a jet of acrid mist (Eisner, '65), the active principle of which is the monoterpene dialdehyde anisomorpal (Meinwald, Chadha, Hurst and Eisner, '62).

The secretory product is elaborated within a pair of integumentary glands which open laterally just behind the head of the insect. Although the gross anatomy of these glands has been described (Eisner, '65), nothing was known of the detailed morphology of their secretory machinery. The process of toxicant production is of particular interest because of the chemical nature of the product, a reactive cyclopentanoid monoterpene. The unusual cyclopentanoid skeleton places anisomorpal in a little-known group of monoterpenes which may serve a protective function in both insects and plants (Eisner, '64). As a terpene, anisomorpal is one representative of a chemical class increasingly implicated as molecular messengers (hormones

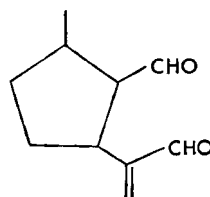


Fig. 1 Anisomorpal.

and pheromones) in a variety of insect systems (Schneiderman and Gilbert, '64; Wilson and Bossert, '63). And finally, manufacture of such a reactive compound by a biological system suggests the classic challenge of toxin production: how does a cell manufacture a poison without poisoning itself? It is the purpose of this paper to describe the structure of the secretory epithelium of the defensive gland of *Anisomorpha* and, by histochemical techniques to follow the production of anisomorpal within this morphological matrix.

MATERIAL AND METHODS

Material

Adult *Anisomorpha buprestoides* (Stoll) were collected in Florida and maintained in the laboratory at Cornell on a diet of

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leaves from locally-available *Quercus* spp. These cultures of produced eggs, but attempts to rear the immatures beyond the second instar were unsuccessful. For all morphological and histochemical studies, the insects were decapitated and the glands dissected free in physiological saline (Griffiths and Tauber, '43).

Morphology

For light microscopy, glands were fixed in alcoholic Bouin's or Helly's fluid and embedded in paraffin wax. Sections, cut at 5–8 μ , were stained with Delafield's hematoxylin, Heidenhain's iron hematoxylin, or Mallory's trichrome. Osmium-fixed glands were stained with ethyl gallate, embedded in agar-ester wax, and cut at 1 μ (Wigglesworth, '57, '59).

Tissues for electron microscopy were fixed in Caulfield's osmium ('57) at pH 7.6, washed in running water, and soaked for one hour in 2% uranyl nitrate (Eisner, McHenry and Salpeter, '64). The tissues were then dehydrated in graded alcohols and embedded in Epon 812 (Luft, '61). Sections, cut with a glass knife on a Porter-Blum microtome, were stained with lead hydroxide (Karnovsky, '61) and photographed at instrument magnifications of 4,000 to 16,000 in the 100 kv beam of an RCA-EMU 3G electron microscope.

Histochemistry

To test for the presence of enzymes, tissues were fixed in cold (0–4°C) formalcalcium or phosphate-buffered formaldehyde (Sabatini, Bensch and Barrnett, '63), embedded in gelatin (Pearse, '60), and cut on a freezing microtome. Other small pieces of tissue were quenched in isopentane cooled by liquid nitrogen, freeze-dried, and vacuum-embedded in paraffin.

Lipids. Frozen sections were flooded with Oil red O in isopropanol (Lillie, '54) or with Sudan black B in ethylene glycol (Chiffelle and Putt, '51). Other glands were chromed according to Baker's acid hematein schedule and then stained with acid hematein (Baker, '46) or with Sudan black B in ethanol. As a control procedure, lipids were extracted with methanol-chloroform (1:2 at 60° for 1–12 hours) or with pyridine (Baker, '46).

Carbohydrates. For glycogen, fixation was at –70°C in Lison's "Gendre" fluid

(Lison and Vokaer, '49). These preparations were stained by the periodic acid-Schiff schedule (Pearse, '60) with the addition of Casselman's ('59) reducing rinse. Control procedure included the omission of the oxidation step and pretreatment with diastase (1% in distilled water). Best's carmine stain for glycogen was also employed (Pearse, '60).

Esterases. Frozen sections were dried on to slides for at least two hours and placed in a moist chamber to incubate at 25°C. Tissues were exposed to naphthol AS-D acetate at pH 7.1 for 30 minutes (Burstone, '57); liberated naphthol was simultaneously coupled with Fast blue RR. For the 5-bromoindoxyl acetate substrate (Holt, '58), the incubation was at pH 8.3 for 1–12 hours; either copper sulphate (10^{-4} M) or potassium ferricyanide/potassium ferrocyanide (10^{-5} M) were employed as oxidation catalysts. Inhibitors, applied for one hour prior to incubation, included the organophosphate paraoxon at 10^{-3} M or silver nitrate at 10^{-2} M. It should also be noted that the metal salts which are employed as oxidation catalysts in the indoxyl procedure may themselves act as esterase inhibitors (Aldrich, '53; Locke, '61).

Phosphatases. Freeze-dried tissues were incubated with the phosphates of naphthols AS-BI and AS-MX at pH's 8.3, 7.3, 6.5 and 5.5; upon hydrolysis these naphthols were simultaneously coupled with Red violet LB (Burstone, '62). For the balance of the phosphatase tests, gelatin sections of formalin-fixed tissues were employed. The naphthols released upon hydrolysis of naphthol AS-BI phosphate and α -naphthyl phosphate were coupled with freshly-prepared hexazonium pararosalin (Barka and Anderson, '62). The Gomori lead nitrate technique, as modified by Holt ('59) was used at pH's 5.2, 7.0 and 8.3. For phosphatases active at pH 7.2, the following standard incubation solution was prepared.

	Final concentration ($\times 10^{-3}$ M)
10 ml Substrate stock solution	2.5
10 ml Tris buffer (0.2 M, pH 7.2)	80
1.5 ml 2% Lead nitrate	3.6
2.5 ml MgSO ₄ or Mg(NO ₃) ₂ or MnCl ₂ (0.1 M)	10

Sites of activity were demonstrated by treating the slides with 1% ammonium sulphide. Substrates included: glycerophosphate, adenosine triphosphate, thiamine pyrophosphate, farnesyl monophosphate, farnesyl pyrophosphate, mevalonic-5-phosphate, and isopentenyl pyrophosphate. The latter two substrates were purchased as the tris-cyclohexammonium salts (Mann Research Laboratories). The salts were dissolved in glass distilled water; the pH raised to 11 with 0.5 N NaOH, and the cyclohexammonium moiety extracted into ether. The aqueous layer was mixed with buffer and the pH adjusted to 7.2 with 0.5 N HCl.

Other phosphatase tests included: adenosine triphosphatase (Wachstein and Meisel, '57), thiamine pyrophosphatase (Novikoff and Goldfischer, '61), inorganic pyrophosphatase (Kurata and Maeda, '56), and alkaline phosphatase (Gurr, '58 after Gomori). To characterize further the enzymes involved, tissues were exposed to inhibitors for one hour prior to incubation with substrate. Cysteine and *p*-chloromercuribenzoate were applied at 2.5×10^{-3} M (Padykula and Herman, '55). NaF and KCN were used at 10^{-2} and 10^{-3} M respectively.

Alcohol dehydrogenase. *Anisomorpha* glands were quenched in isopentane (cooled by dry ice-acetone), frozen on to the stage of a freezing microtome, and sectioned at 25 μ . As each section thawed on the slide, it was flooded with incubation medium. Media, mixed fresh for each incubation, were based upon the general technique of Hess, Scarpelli and Pearse ('58), with the exception that substrates were added in dimethylformamide. Tissues were incubated for 15 minutes at 37°C, washed with distilled water, and post-fixed for at least 30 minutes in formaldehyde.

MORPHOLOGY

Light microscopy

The two defensive glands of *Anisomorpha* open on either side of the prothorax. By removing the thoracic dorsum, one can trace the heavily-muscled glands as they run back above the gut into the mesothorax. Each gland consists of a cuticular sac surrounded by inner circular

and outer longitudinal muscles; by compressing the sac these muscles expel the defensive secretion. Beneath the muscle layers and coating the much-folded surface of the secretion reservoir is the glandular epithelium, 20–30 μ in thickness, which is responsible for elaboration of the defensive toxicant (fig. 2).

Iron hematoxylin demonstrates two distinct populations of nuclei in the glandular epithelium (fig. 3). Ovoid nuclei, ca. $3 \times 7 \mu$, are packed along the cuticular intima of the secretion reservoir while larger, more-rounded nuclei are found at intervals in the basal zone of the epithelium. Although cell boundaries are not visible in the photographs, the distribution of the two types of nuclei suggests that the ovoid nuclei are within a layer of cytoplasm-poor epithelial cells while the rounded nuclei are within a layer of larger secretory cells.

Specialized secretory regions are present in the basal zone of the glandular epithelium and are most-clearly seen in Mallory's stained preparations. Each appears as an array of light-blue fibrillae radiating out into the surrounding cytoplasm from a weakly-staining central region (fig. 4). From this center, a fine efferent ductule runs through the cell, winds about in the epithelial layer, and finally traverses the cuticle of the reservoir. In squash preparations, ductules up to 45 μ in length were seen. The resistance of these ductules to aqueous potassium hydroxide indicates that like the reservoir itself, they are cuticular (fig. 5).

Numerous pigment granules are found within the epithelial cells and along the efferent ductules as they run toward the specialized secretory regions (fig. 6). Although the chemical identity of the pigment has not been rigorously established, its capacity for reversible oxidation-reduction² suggests that it is an insectorubin, such as described by Fuzeau-Braesch ('57) in the epidermis of other phasmids. The fact that these epithelial cells are continuous with the epidermal cells of the body

² In fixed tissues, the pigment granules are insoluble in water, ethanol, acetone, or xylol; they contain reducing groups (ferric-ferricyanide test; Lillie, '54); they can be bleached by 10% hydrogen peroxide and subsequently demonstrated with ammoniated silver nitrate. The Nile blue sulphate test for melanin (Gurr, '62) was negative.

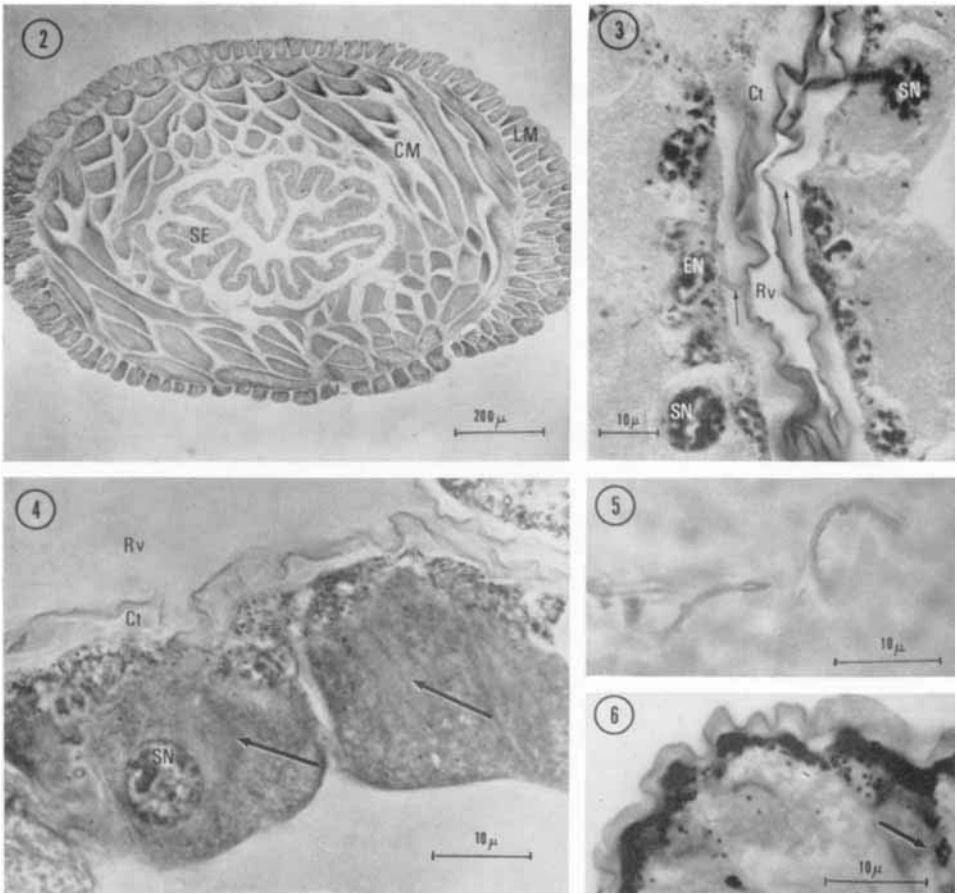


Fig. 2 Transverse section through the defensive gland of *Anisomorpha* showing the secretory epithelium (SE) inner circular (CM) and outer longitudinal muscles (LM). Bouin's paraffin-embedded, Mallory's trichrome.

Fig. 3 Section through the secretory epithelium. Several nuclei of secretory cells (SN) and squamous epidermal cells (EN) are shown. Efferent ductules (arrows) traverse the reservoir cuticle (Ct) in passing from the squamous cell layer to the reservoir (Rv). Bouin's, paraffin-embedded, Heidenhain's hematoxylin.

Fig. 4 In each of the two adjacent secretory cells, the secretory apparatus can be seen (arrows). Note also the nucleus of one cell (SN), the cuticle (Ct), and the reservoir (Rv). Bouin's, paraffin-embedded, Mallory's trichrome, phase-contrast.

Fig. 5 Two cuticular efferent ductules after KOH treatment. Whole mount.

Fig. 6 Transverse section through epithelium after ferric-ferricyanide test. The pigment granules are deeply stained and at the arrow can be seen to extend into the secretory cell layer. Absolute ethanol, paraffin-embedded.

cuticle which contain similar pigment granules implies a common origin (and perhaps chitogenic function) for these cells.

Electron microscopy

The low-power electron micrographs (fig. 7) are in agreement with the picture

of cell relationships already deduced from light microscopy. Along the cuticular intima of the secretion reservoir is a layer of squamous epithelial cells within which are pigment granules and the winding efferent ductules. Beneath the squamous cells are the larger secretory cells whose most prominent features are the radiating

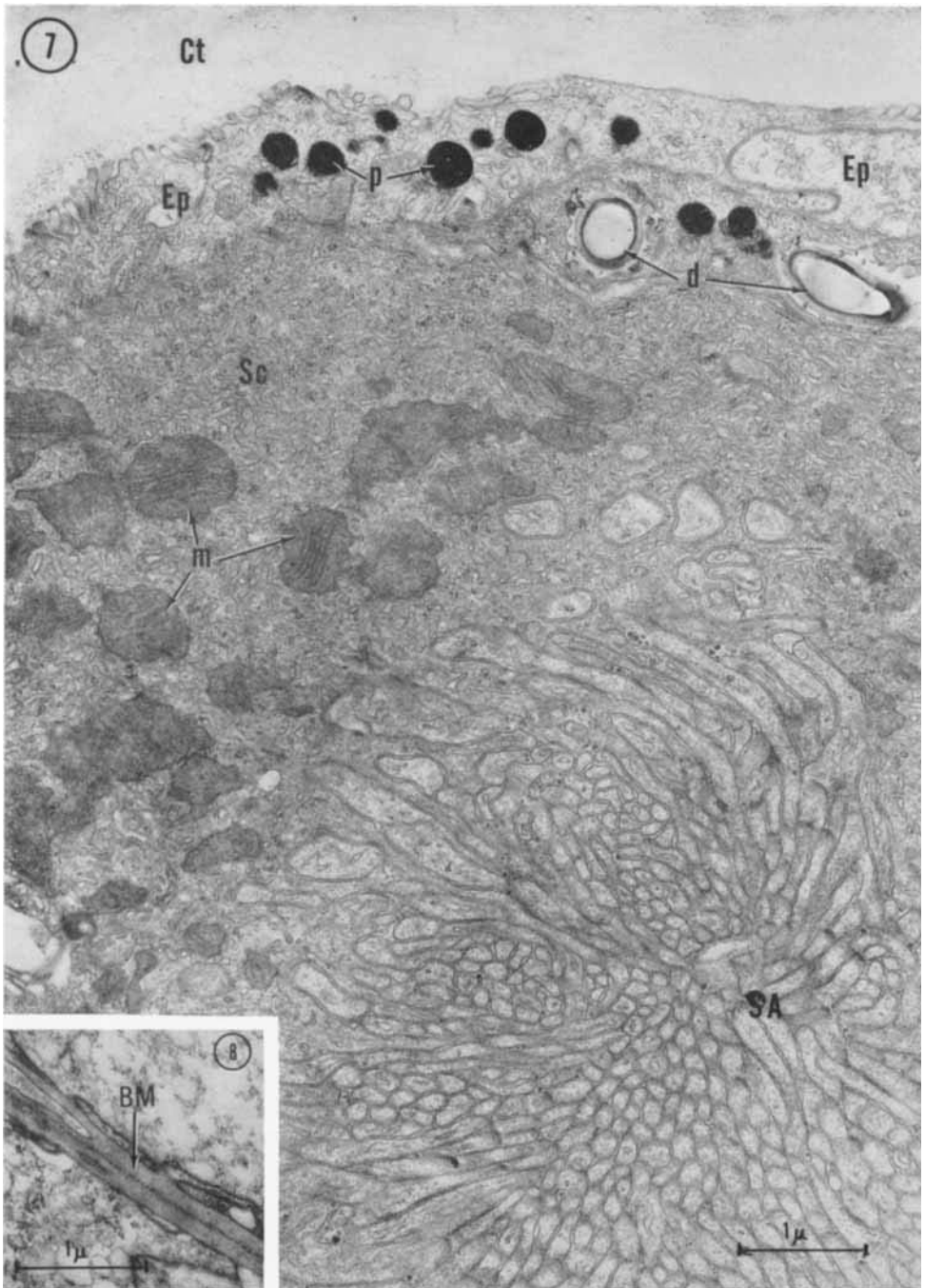


Fig. 7 Low power survey electron micrograph of the secretory epithelium. Two squamous epithelial cells (*Ep*) lie against the cuticle of the reservoir (*Ct*). The cytoplasm, rich in pigment granules (*p*), is penetrated by cuticular ductules (*d*). At its periphery the secretory cell (*Sc*) contains numerous mitochondria (*m*) and at the center of this cell lies the secretory apparatus (*SA*).

Fig. 8 Basement membrane (*BM*) between the basal margins of two secretory cells.

arrays of membranes of the specialized secretory regions, henceforth to be called the "secretory apparatus." Other features of these cells can be seen in the micrographs (figs. 7 and 8) and in the diagram (fig. 9).

Both the cuticle of the gland reservoir and that of the efferent ductule contain similar structural components (cuticulin, homogenous inner epicuticle, and endocuticle, in the sense of Locke ('65)); however, the endocuticle of the reservoir is considerably thicker. In both, lamellate endocuticle is successively overlain by homogenous epicuticle (ca. 80 m μ in thickness) and an electron-dense cuticulin (ca.

30 m μ in thickness). The lamellae of the endocuticle are more prominent in the reservoir (fig. 10), and where a ductule passes through the reservoir wall, the microfibrils tend to encircle the ductule. Within and immediately adjacent to the homogenous layer of the ductule are short osmiophilic filaments of unknown function (fig. 10). In the glands of recently-molted *Anisomorpha*, the microvilli from the epithelial cells project into the lower granular zone of the endocuticle (fig. 10).

Cytoplasmic processes of the squamous epithelial cells follow the efferent ductules as they pass toward the secretory apparatus. The descending tongues of cytoplasm

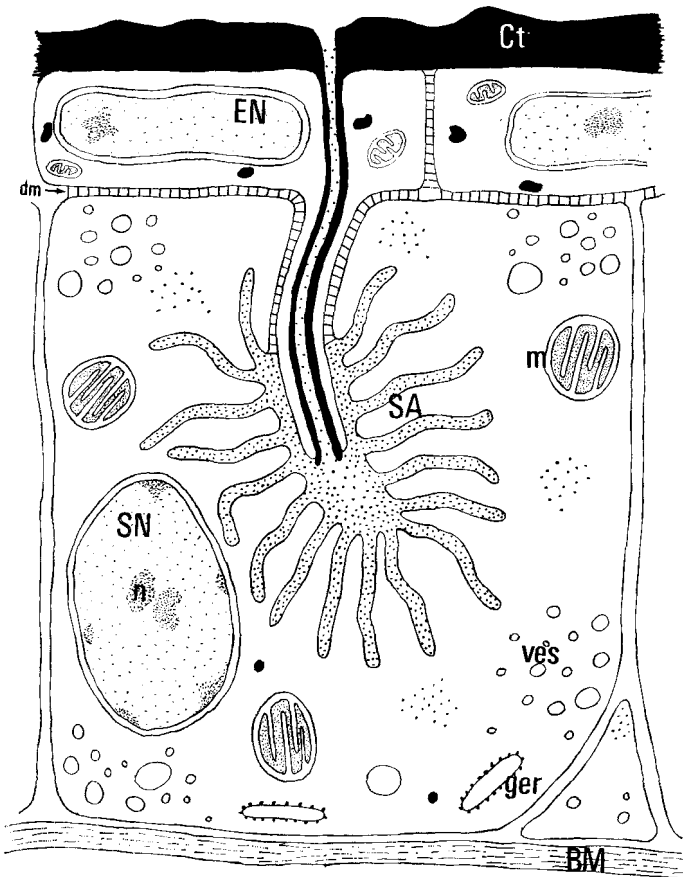


Fig. 9 Simplified reference diagram of the cells in the secretory epithelium. BM, basement membrane; Ct, cuticle of the reservoir; dm, desmosomes; EN, nucleus of squamous epithelial cells; ger, granular endoplasmic reticulum; n, nucleolus of secretory cell; m, mitochondria; SA, secretory apparatus; SN, nucleus of secretory cell; ves, agranular vesicles. Pigment granules, the efferent cuticular ductule, and mitochondria are also shown within a squamous cell.

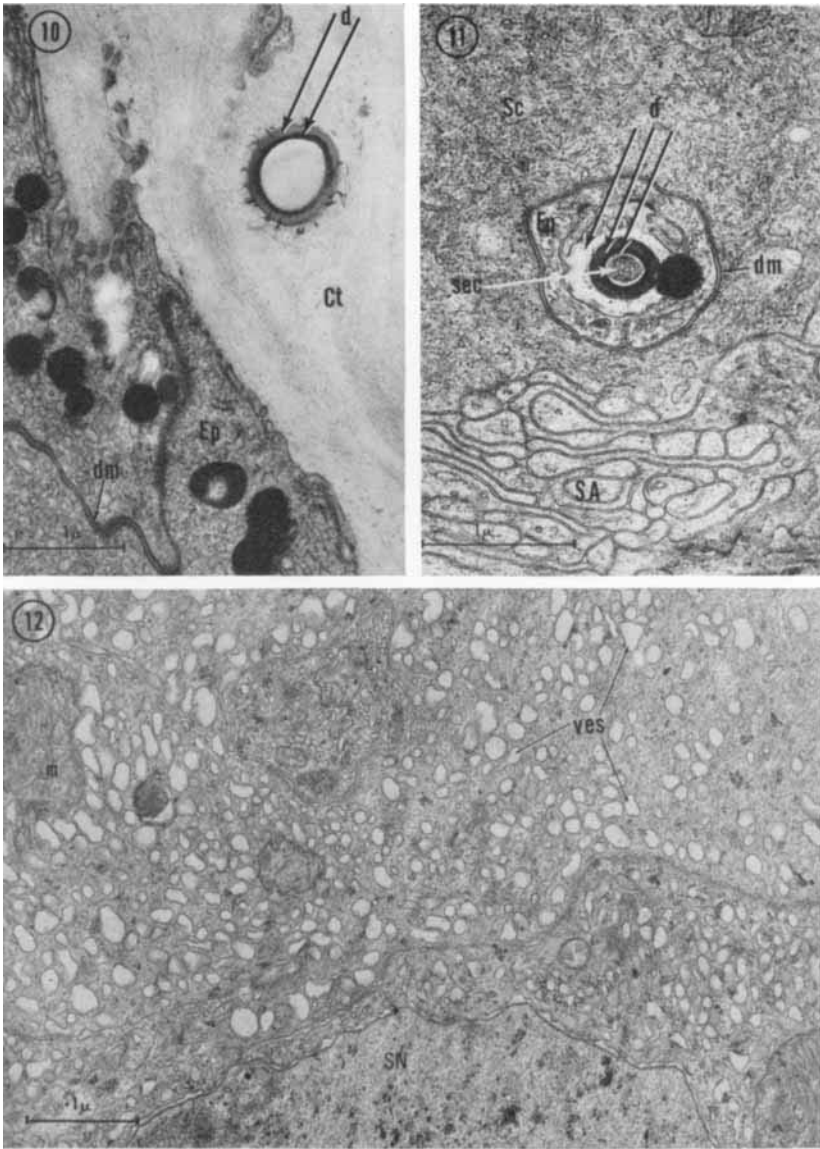


Fig. 10 Efferent cuticular ductule (*d*) penetrates the cuticle of the reservoir (*Ct*). Note the concentric layers of the ductule (arrows) and the surrounding filaments. Desmosomes (*dm*) join the plasma membranes of two squamous epithelial cells (*Ep*).

Fig. 11 Cuticular ductule (*d*) surrounded by a sheath of epithelial cell cytoplasm (*Ep*) as it penetrates a secretory cell (*Sc*). Note the three layers of the ductule (arrows) and the secretion (*sec*) in its lumen. The two cells are linked by desmosomes (*dm*). A secretory apparatus (*SA*) lies below the ductule.

Fig. 12 Cytoplasm of a secretory cell showing mitochondria (*m*) and the electron-transparent vesicles (*ves*). The nucleus of another secretory cell (*SN*) is also seen.

which surround the ductules may contain characteristic pigment granules and small mitochondria, and invariably they are bonded by intracellular bridges (septate desmosomes) to the plasma membrane of the secretory cell (fig. 11). Similar desmosomes join squamous epithelial cells to each other in the more superficial layer of the glandular epithelium (fig. 7).

In contrast to the squamous epithelial cells, the secretory cells are large (up to 30 μ across) and more rounded. Near the center of a cytoplasm rich in formed elements is the nucleus with its conspicuous nucleoli and scattered chromatin. The peripheral cytoplasm contains mitochondria set in a granular matrix of moderate electron density (figs. 7 and 12) and large numbers of electron-transparent vesicles, 200–300 μ in diameter (fig. 9; also figs. 7 and 12). These vesicles may have contained non-osmiophilic lipid in the living cell. Lysosome-like bodies are also found around the margins of the secretory apparatus.

The secretory apparatus is an array of microvilli converging on a central cavity about 300 $m\mu$ in width (fig. 13). Within each microvillus is an occasional small electron-transparent vesicle but no mitochondria are present. Irregular deposits of osmiophilic material lie within the central cavity (figs. 14 and 15) and within the efferent ductule (see also fig. 11). These deposits apparently represent the secretory product, which reacts (darkens) upon exposure to the osmium fixative *in vitro*. The epicuticular tip of the efferent ductule, free of its surrounding sheath of cytoplasm and without apparent endocuticular layers, projects into the central cavity (fig. 14). At this point secretion can be seen within its open end. Figure 16 provides a diagrammatic reconstruction of the microvilli converging upon the central cavity.

Both adult and nymphal *Anisomorpha* possess defensive glands, similar in gross morphology and in fine structure. The full secretory cycle within any one gland is complex and is beyond the scope of the present study. However, it is clear from our work that the secretory cycles of the various cells are asynchronous: adjacent to a cell such as that shown in figure 7 may be another whose microvilli are

clumped together and surrounded by myelin figures, degenerating mitochondria, and numerous lysosomes. This impression is supported by light microscopy with osmium-ethyl gallate preparations in which one secretory apparatus may appear membranous while an adjacent apparatus appears merely as a cluster of large vesicles. Somewhat analogous functional differences have been reported by Stein ('62) in his electron microscopic study of the terpene-producing glands of *Bombus*.

HISTOCHEMISTRY

The glandular epithelium is poor in glycogen and ribonucleic acid but rich in lipids. Both the periodic acid-Schiff's schedule and Best's carmine produced only a faint cytoplasmic staining which persisted even in sections pretreated with diastase or saliva. In contrast, lipids abound in the cytoplasm of the secretory cells. Oil red O for neutral fats revealed minute fat droplets which could be removed by methanol-chloroform extraction (fig. 20). Following chromation, both the acid hematein technique (fig. 17) and Sudan black B in ethylene glycol demonstrated similar lipid deposits. Presumably these granular deposits correspond to the electron-transparent vesicles described earlier. Chromation also "unmasked" another lipid concentration, the membranes of the secretory apparatus. These membranes could be demonstrated with acid hematein (blue-black) and also with Sudan black B in ethanol (figs. 17–19).

Esterases

As in most animal cells, carboxylic esterases are diffusely scattered throughout the cytoplasm of the secretory cells. This general cytoplasmic activity can be seen following either the naphthol-AS or the indoxyl (copper sulphate catalyst) techniques (fig. 26), and was prevented by preincubation with paroxon or silver nitrate or by the use of ferricyanide/ferrocyanide as oxidation catalyst in the indoxyl technique. In addition, the naphthol substrate demonstrated a paroxon-resistant esterase at discrete locations whose size and distribution correspond to that of the secretory apparatus (fig. 27). This

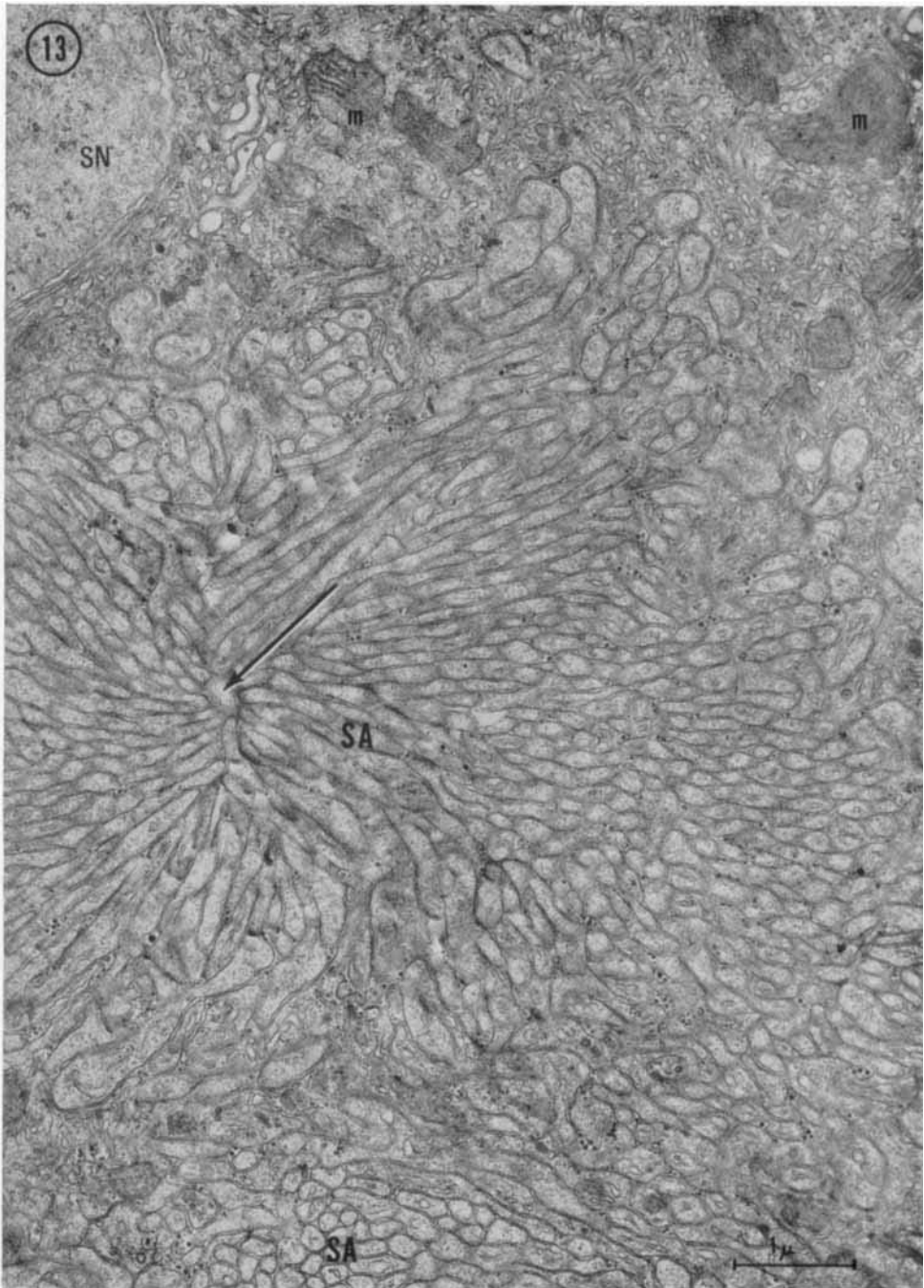


Fig. 13 The microvilli of the secretory apparatus (SA) converge toward the central cavity (arrow) which contains secretion.

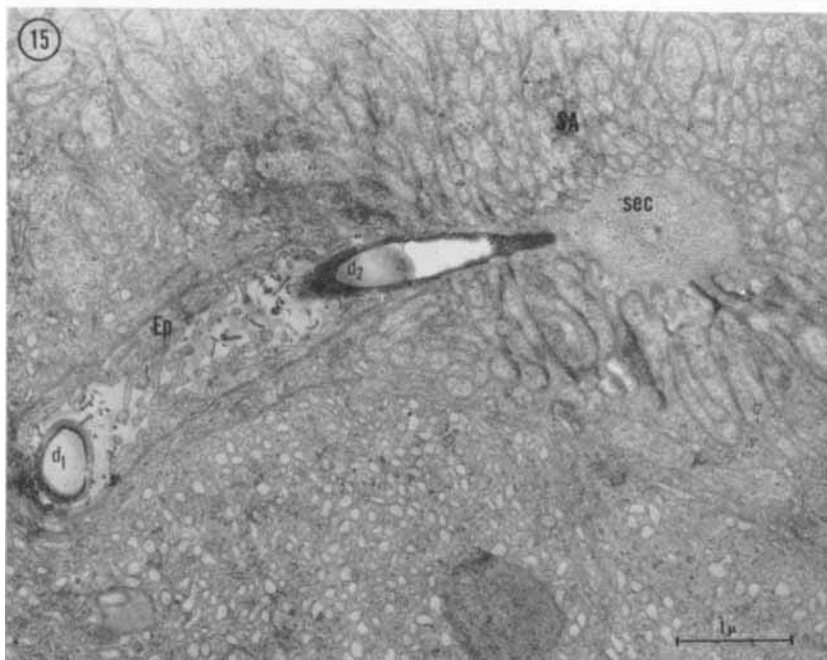
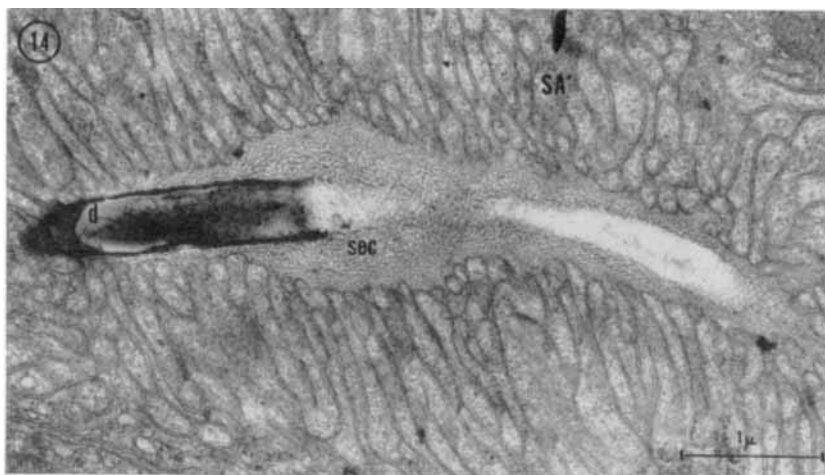


Fig. 14 The efferent cuticular ductule (d) at the center of the secretory apparatus (SA). Secretion lies in the central cavity.

Fig. 15 The efferent cuticular ductule cut transversely (d_1) as it approaches the central cavity within the secretory apparatus. The surrounding layers of squamous epithelial cytoplasm are absent as it nears the secretion (sec) in the central cavity; only the epicuticle (mostly cuticulin at this level) persists (d_2).

paraoxon-resistant enzyme was inhibited by pretreatment with silver nitrate.

Phosphatases

A wide variety of phosphate-esters are hydrolyzed by enzymes in the secretory

cells of the glands. The esters split include: glycerophosphate, adenosine triphosphate, naphthol AS-BI and AS-MX phosphates, α -naphthyl phosphate, mevalonic-5-phosphate, and isopentenyl pyrophosphate (figs. 21–25). In the cytoplasm

of the secretory cells, an acid phosphatase could be detected at localized points suggestive of the lysosome-like bodies described in the electron micrographs. This enzyme was inhibited by sodium fluoride and was most active at pH's 5.0 to 5.5; at higher pH's its activity was diminished (pH 7) or abolished (pH 8), as shown in figures 21 to 24.

Other phosphatase activity, resistant to sodium fluoride, was associated with the secretory apparatus itself (figs. 23-25). With all substrates, activity was highest at this site when the pH of the incubation medium was near neutrality. As demonstrated by experiments with a series of

inhibitors (see table 1), at least two enzymes were responsible for the hydrolysis. With other substrates hydrolyzed at this location (glycerophosphate, naphthol phosphates, adenosine triphosphate) the pattern of inhibition resembled that of isopentenyl pyrophosphate. For all these tests, either magnesium (10^{-3} M) or manganese (10^{-4} M) was necessary as a co-factor.

No hydrolysis of any phosphate-esters was detected at pH 9 or above, nor was the hydrolysis of thiamine pyrophosphate, farnesyl monophosphate, farnesyl pyrophosphate, or sodium pyrophosphate demonstrated at any pH.

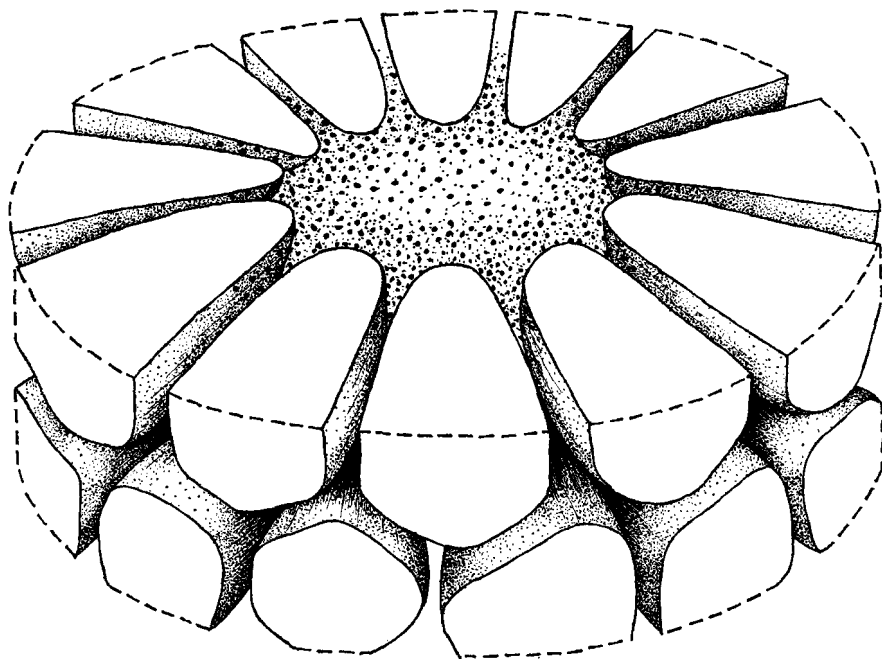


Fig. 16 Reconstruction of the center of the secretory apparatus. Microvilli surround the central cavity which contains coarsely stippled secretion.

TABLE 1
Phosphatase activity associated with the secretory apparatus

Substrate	Treatment						Substrate blank
	No inhibitor	pCMB	Cysteine	NaF	KCN	60°C 1 hr.	
Mevalonic-5-phosphate	3	0	1	3	1	0	0
Isopentenyl pyrophosphate	3	3	0	3	3	0	0

Activity rated from 0 (none) to 3 (high).

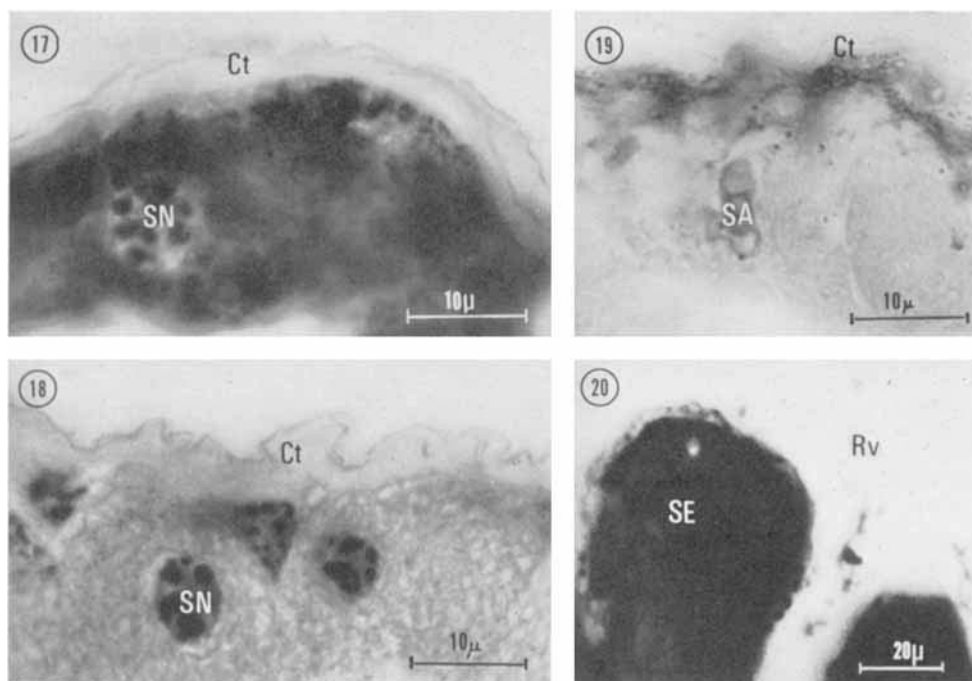


Fig. 17 Acid hematein test showing the deposits of lipids throughout the secretory cell layer. The lighter zone near the cuticle of the reservoir (Ct) reflects the lower lipid content of the squamous epithelial cells. Formol-calcium, chromed, gelatin.

Fig. 18 Acid hematein test (see fig. 17), pyridine-extracted control. Baker's weak Bouin's, chromed, gelatin.

Fig. 19 Sudan black B in 70% ethanol. Concentration of lipids, presumably phospholipid of the microvilli, are associated with the secretory apparatus (SA). Formol-calcium, chromed, gelatin.

Fig. 20 Oil red O for neutral fats showing the dense fat deposits in the secretory epithelium. Formol-calcium, gelatin.

Alcohol dehydrogenase

Pyridine nucleotide-linked alcohol dehydrogenases are present in fresh-frozen sections of *Anisomorpha* glands. Although the poor morphology in such sections prevented precise intracellular localization, the concentrated activity in the glandular epithelium (fig. 28) and some characteristics of these enzymes could be demonstrated. The results, summarized in table 2, suggest that the alcohol dehydrogenase is preferably NADP-linked and geraniol is the most efficiently dehydrogenated of the substrates tested.

DISCUSSION

The production of defensive toxicants such as anisomorpal can be visualized as

occurring in two sequential stages: a first stage, involving the building of the molecular framework by reliance upon widespread biochemical pathways, and a second stage, in which this molecular framework is converted, by relatively minor

TABLE 2
Alcohol dehydrogenase

Substrate	Cofactor	
	NAD	NADP
Geraniol	2	3
Citronellol	1	1
Nerol	1	2
Butanol	1	1
Ethanol	1	1
Blank	0	0

Activity rated from 0 (none) to 3 (high).

chemical modifications, into the toxic end-product itself.

Production of the terpene skeleton

Among the Insecta, polycyclic steroids usually must be derived from preformed dietary sources (Clayton, '64; House, '65), but smaller terpenes, such as the citronellal isolated from an ant (Happ and Meinwald, '65), anisomorphal (Meinwald, Happ, Labows and Eisner, '66), or the farnesol isolated from silkworm caterpillars (Schmialek, '63) can be synthesized *de novo* from acetate or mevalonate. On the assumption that the production of the cyclopentanoid anisomorphal is via the classical mevalonic acid pathway, its synthesis should occur in three major segments: (1) the formation of mevalonic acid, (2) the conversion of mevalonic acid into isopentenyl pyrophosphate, and (3) the joining of two "active isoprenes" to form geraniol followed by cyclizing to yield a five-membered ring.

Mevalonic acid is derived from hydroxymethylglutaryl-coenzyme A which is itself the product of the coupling of three acetyl-coenzyme A's. One source of such two-carbon acetate units in the *Anisomorpha* secretory cells is the abundant lipid reserves which (at least in the case of neutral fats) may be mobilized by the action of esterases. Esterases (lipases) have been demonstrated in the fat body and flight muscles of insects where they are presumed to hydrolyze neutral lipids to yield the fatty acids burned in flight (George, Vallyathan, and Sciara, '58; George and Eapen, '59). The paraoxon-resistant esterase associated with the secretory apparatus might play a similar role; alternatively these enzymes might be concerned with the production of the hydrocarbon components of the secretion.

Conversion of mevalonic acid, formed from these acetates, into isopentenyl pyrophosphate is effected by several biochemical steps. The first is catalyzed by mevalonic kinase and involves phosphorylation of the alcohol, as shown in figure 29. Even a cursory inspection of this reaction suggests that the phosphatase hydrolyzing mevalonic-5-phosphate in the histochemical system might merely be mevalonic kinase, acting from right to left. Although

no enzymes of the mevalonic acid pathway have yet been isolated from insect material, a comparison of the "mevalonic-5-phosphatase" with mevalonic kinase described from liver (Goodman and Popják, '60; Levy and Popják, '60) and yeast (Tchen, '58) reveals striking similarities. Like the *Anisomorpha* enzyme, these mevalonic kinases have pH optima around neutrality, are inhibited by *p*-CMB but not by sodium fluoride, and require magnesium or manganese as cofactor. Certainly one would not expect this kinase to act as a phosphatase in living tissue, since the loss of a phosphate without regeneration of a high-energy bond is energetically wasteful. But in the histochemical system, mass action forces the reaction to the left: free phosphate ions are immediately precipitated as the lead salt and thus removed from an equilibrium equation. A similar correlation between hydrolytic action *in vitro* by an enzyme with synthetic action *in vivo* has been suggested by Lardy and Wellman ('53) for mitochondrial adenosine triphosphatase.

At least one more phosphatase, hydrolyzing a variety of substrates, is associated with the secretory apparatus and possibly is concerned with the production of the secretory product. The exact roles of such enzymes in the production of anisomorphal are uncertain. They could act to remove phosphates from geranyl phosphate and thus lead to the formation of geraniol; they could function as metabolic control mechanisms, limiting the synthetic activity of the mevalonic acid pathway by tearing down allyl phosphates as fast as these are produced; or perhaps they might merely be one of the many phosphatases frequently associated with secretion across biological membranes (for examples see Ashworth, Luibel and Stewart, '63).

In formation of the cyclopentane ring, an acyclic terpene produced by the mevalonic acid pathway must somehow be cyclized. Biogenetic schemes for this step involve the biochemical equivalent of an intramolecular Michael reaction, and in fact, Sir Robert Robinson and his co-workers have synthesized iridodial and *iso*-iridomyrmecin from citronellal by this route (Clark, Fray, Jaeger and Robinson, '59). Such a hypothesis presupposes that

the acyclic precursor must be an aldehyde rather than the alcohol (geraniol) which is the immediate product of the mevalonic acid pathway. Within the glandular epithelium of *Anisomorpha*, an enzyme effecting the dehydrogenation of terpene alcohols to aldehydes has been demonstrated. Further, this particular enzyme is unlike many alcohol dehydrogenases (Racker, '55) both in its activity toward terpene alcohols and in its preference for NADP as the cofactor.

Although the histochemical results do not demonstrate every step in the production of anisomorpha, they do lend strong support for the view that the mevalonic acid pathway is the actual biogenetic route. More direct evidence for the presence of this pathway in another insect (*Acanthomyops claviger*) has been presented in an earlier paper (Happ and Meinwald, '65).

Production of a defensive toxicant

It is a formidable task for a secretory system to produce such reactive molecules as anisomorpha without poisoning its own biosynthetic machinery. Schildknecht and Holoubek ('61) have shown that in the quinone-producing defensive glands of *Brachynus*, oxidation of diphenol to quinone does not occur in the secretory cells but rather in a special compartment of the secretion reservoir. On the basis of this result and his own work on *Apheloria* (HCN-producing millipede) and *Eleodes* (quinone-producing beetle), Eisner has suggested that analogous extracellular reaction compartments may be present in many toxicant-producing glands. In the case of *Apheloria*, the final step in HCN-production occurs in a special vestibule of the gland reservoir (Eisner, Eisner, Hurst, Kafatos and Meinwald, '63), but in *Eleodes*, the extracellular reaction compartments are associated with each secretory cell complex (Eisner, McHenry and Salpeter, '64; Happ and Eisner, in preparation). Relatively nontoxic precursors are secreted out of the cytoplasm of the secretory cell; their conversion to a reactive quinone is effected within the elaborate system of vesicles and ductules described by Eisner, McHenry and Salpeter ('64).

A recent study clearly demonstrates that the lipid constituents of plasma membranes vary widely in different mammalian cell types, indicating that "there is both tissue specificity and functional specificity in the organization of cellular membranes" (Ashworth and Green, '66). Although we have no direct evidence to support this suggestion, it is conceivable that opposite surfaces of secretory cells might possess differing lipid content, reflecting their functional specialization. In the case of extracellular reaction compartments lined by plasma membranes in insect defensive glands, the membrane lining of the compartment may be particularly resistant to toxic reaction products formed within it.

Although we have not found such dramatic morphological compartments in the glands of *Anisomorpha*, it is certainly possible that the narrow central cavity within the secretory apparatus may serve as such an extracellular reaction chamber. However, none of the light histochemistry has

Fig. 21 α -Naphthol phosphatase at pH 5.0. Activity is highest around the secretory apparatus (SA) and in particulate masses in the surrounding cytoplasm. Formol-calcium, gelatin, coupled with diazotized hexammonium pararosalin.

Fig. 22 Gomori test for acid phosphatase. Activity similar to figure 21. Formol-calcium, gelatin.

Fig. 23 Gomori test for acid phosphatase, NaF inhibited. Although the particulate reaction is almost totally inhibited, that associated with the secretory apparatus (SA) persists. Formol-calcium, gelatin.

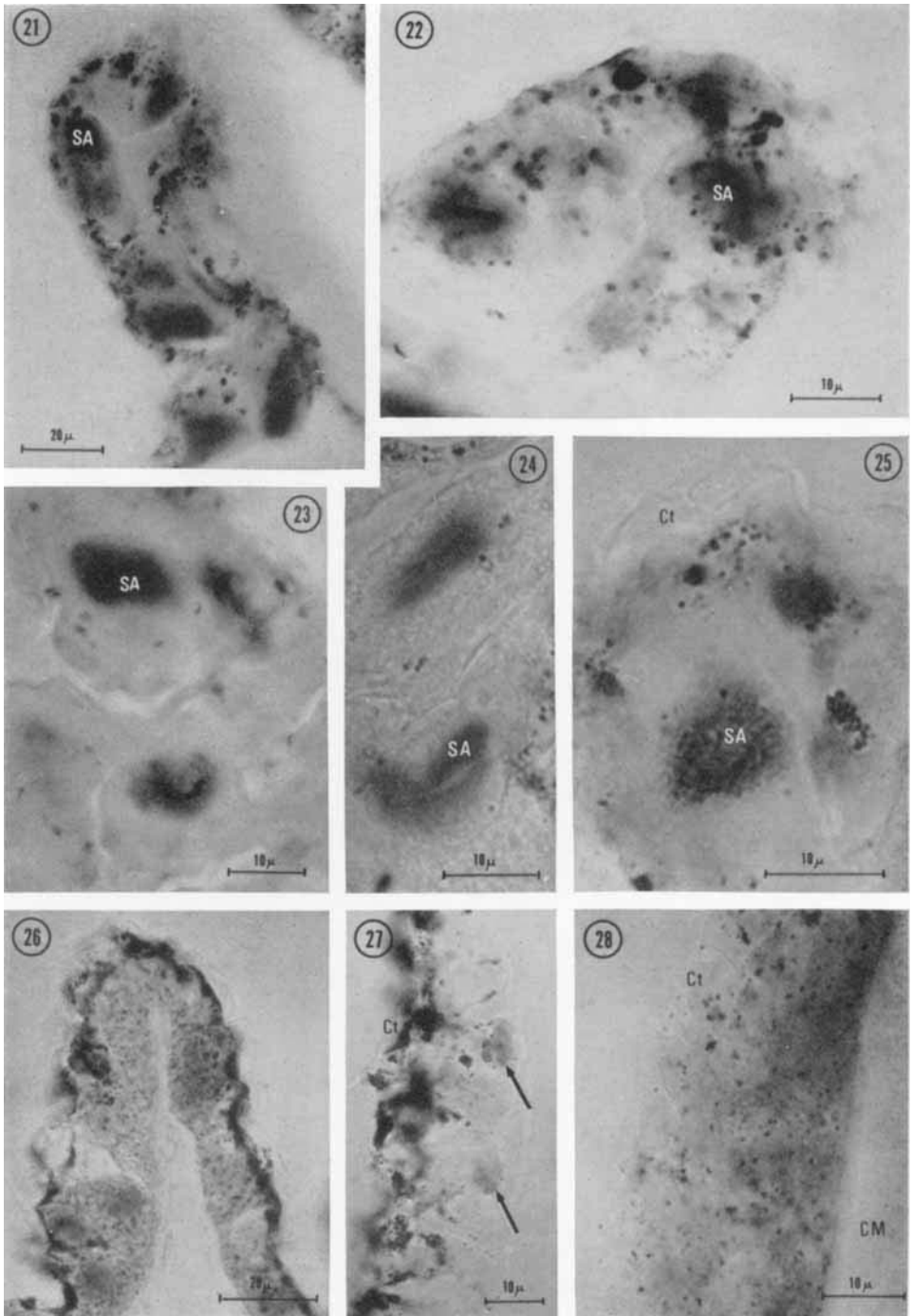
Fig. 24 Naphthol AS-MX phosphatase (pH 8.3). Only the reaction in the secretory apparatus persists (SA). Freeze-dried, paraffin, coupled with Red violet LB.

Fig. 25 Hydrolysis of 5-phosphomevalonic acid, lead nitrate technique (pH 7.2). The reaction is confined to the secretory apparatus. Formol-calcium, gelatin.

Fig. 26 Indoxyl acetate test for carboxylic esterase. Formol-phosphate, gelatin.

Fig. 27 Naphthol AS-D acetate test for carboxylic esterase. The blue azo dye is deposited in patches (arrows) which may represent the secretory apparatus. Formol-phosphate, gelatin, coupled with Fast blue RR.

Fig. 28 Geraniol dehydrogenase by Nitro BT technique, TPN cofactor. The reaction is highest in the secretory epithelium and almost absent from the cuticle of the reservoir (Ct) and the adjacent musculature (CM). Fresh frozen section.



Figures 21-28

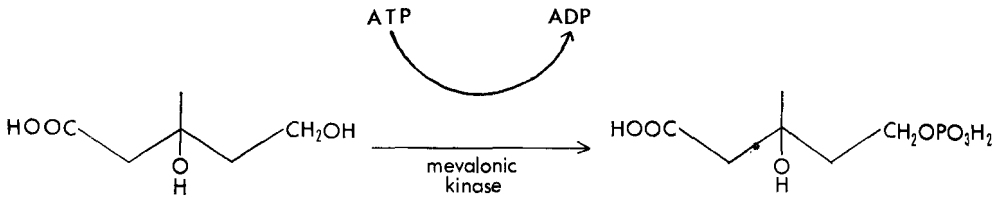


Fig. 29 Mevalonic kinase.

provided precise localization of any reaction step in this narrow central cavity. Further, the biochemistry of the terminal cyclization steps in the synthesis of anisomorphal is completely unknown. Until the final steps in the biosynthesis of cyclopentanoid monoterpenes are better understood and this knowledge applied in a histochemical study (probably at the electron microscope level) of the glands of *Anisomorpha*, an assignment of sequential reactions to sequentially-arranged compartments in this system must remain only an attractive speculation.

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