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NJIDDA M. GADZAMA*† and GEORGE M. HAPP*‡

THE STRUCTURE AND EVACUATION OF THE SPERMATOPHORE OF *TENEBRIO MOLITOR* L. (COLEOPTERA: TENEBRIONIDAE)

ABSTRACT. Sperm are transferred from male to female mealworm beetles within a spermatophore. The highly organized spermatophore can be visualized as a tube, blind at both ends, and invaginated at the anterior. The wall of the tube is composed of two layers, one of fibrous protein and the other of lipoprotein which appears frothy in electron micrographs. After being deposited in the bursa of the female, or placed in saline, the spermatophore undergoes sequential stereotyped expansions at the anterior tip and then bursts, releasing semen within the female bursa. The 'evacuated' spermatophore becomes coated by a transluscent layer and is ejected from the female 18 hr after copulation.

Introduction

IN certain invertebrates, sperm are packaged for transfer from male to female. Sperm sacs or spermatophores have been found in a wide variety of insect species which employ internal fertilization (references in Davey, 1960, 1965; Englemann, 1970; Gerber, 1970; Schaller, 1971; Wigglesworth, 1965). Spermatophores vary in overall shape and in internal organization, depending on their particular role in sperm transfer for each species. Perhaps the most interesting aspect of these structures is that they are assembled, usually from products of male accessory glands, in extracellular spaces. The assembly process, and the relative contributions of the various glands, have recently been studied experimentally in a grasshopper (*Gomphocerus rufus*) by Hartmann (1970). It is our intent to analyze the process of sperm transfer, including the details of structure and assembly of the spermatophore in the mealworm beetle, *Tenebrio molitor*.

Although spermatophores were once considered rare in Coleoptera (Kalifa, 1949b), they have been demonstrated recently in several families including the Tenebrionidae (Fiori, 1954; Davey, 1960; Gerber, 1970). In the case of *Tenebrio*, the spermatophore was described as a long narrow tube, encased in a scanty gelatinous matrix (Davey, 1960; Jones, 1967), and showing definite structure, namely a 'plug' at its anterior end (Jones, 1967). No information was available on the fine structure of this package, its rupture, nor on its formation. In the present paper, we will concentrate on the histochemical and ultrastructural examination of the walls of the tube and a description of the process of sperm liberation. To our knowledge, this is the first paper describing the ultrastructure of an insect spermatophore.

* Department of Biology, New York University, Bronx, New York 10453, U.S.A.
† Department of Biological Sciences, Ahmadu Bello University, Samuru, Zaria, Nigeria.
‡ Department of Zoology and Entomology, Colorado State University, Fort Collins, Colorado 80521, U.S.A.

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Materials and Methods

Tenebrio molitor L. were obtained from a laboratory stock culture, maintained on chicken feed (Purina Startena) and sliced potato. The insects were sexed at the pupal stage, and males and females were segregated for about 1 week after ecdysis. Mating

occurred promptly when males were allowed access to females, and spermatophore transfer required only 3 to 5 min. The spermatophores could be retrieved by two methods; either by separating male and female *ca.* 30 sec after the onset of copulation (in which case the spermatophore protruded from the intromittant organ of the male) or by allowing normal copulation to proceed and then removing the spermatophore from the bursa of the female after some convenient intervening time. All dissections were performed in *Tenebrio* saline (Butz, 1957).

Light microscopy

Fresh preparations of sperm sacs retrieved from males and females were placed in cold saline solutions and observed in phase contrast or dark-field microscopy. The contents of the spermatophore were squeezed out and checked for live sperm and extracellular products.

Specimens were fixed by various methods including 10% neutral formalin, cold formol-calcium, and aqueous and alcoholic Bouin's. Specimens for frozen sections were embedded in O.C.T. medium (Lab-Tek product) and sections 12 μ thick were obtained on an IEC cryostat. Other spermatophores were embedded in paraffin (M.P. 56°C) and sectioned at 6 μ. The paraffin sections were stained with alcoholic bromphenol blue (Bonhag, 1955), and 1-fluoro 2,4-dinitrobenzene (DNFB) for protein; periodic acid–Schiff's (Lille, 1955), and alcian blue (Pearse, 1968) for polysaccharides and mucopolysaccharides; and Oil red O and Sudan black B (Lillie, 1965; Pearse, 1968) for lipid deposits.

Electron microscopy

Spermatophore pieces were fixed in 5% glutaraldehyde in 0·1 M phosphate buffer at pH 7·4 for 4 hr, washed in four changes of phosphate buffer (pH 7·4) with 10% sucrose added and then post-fixed in 1% osmium tetroxide in phosphate buffer pH 7·4 at 0–4°C (Locke, 1966). Fixed spermato-

Fig. 1. Ventral view of the posterior of male *Tenebrio* showing a spermatophore (Spph) protruding out of the male gonopore 25 sec after the beginning of copulation. Fresh preparation; whole mount. ×10.

Fig. 2. Anterior segment of the spermatophore. The core (C) is clearly seen. Formalin; whole mount; phase contrast. ×84.

Fig. 3. Spermatophore left 2 hr in osmium tetroxide. The A_2 (central material) of the core 'stains' black indicating the presence of lipid. Whole mount. ×46.

Fig. 4. Cross-section of spermatophore anterior. The lumen (L) contains deeply stained semen (S). Three layers of the core are designated as A_2, B_3, and B_4. Bouin's; Lower's trichrome. ×300.

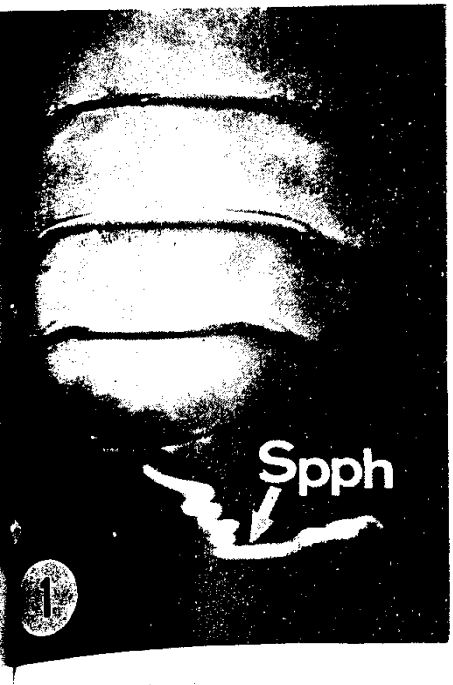
Fig. 5. Longitudinal section of the spermatophore tube wall showing the layers of A_1, B_1, and B_2. Glutaraldehyde osmium tetroxide; toluidine blue. ×2550.

Fig. 6. Anterior segment of the spermatophore (longitudinally sectioned) showing the various layers. The B layers of the core and of the tube wall appear to be continuous. Formalin; Sudan black B. ×270.

Fig. 7. An oblique section of the core showing the A_2 layer most deeply stained. Formalin; Sudan black B. ×270.

Fig. 8. Spermatophore treated with sodium hypochlorite to test for the presence of masked lipid. The contents of the lumen appear bleached but the A layers, particularly A_2 are stained. Formalin; NaC10; Sudan Black B. ×384.

Fig. 9. Longitudinal section of the spermatophore stained for lipid. The central material (A_2) of the core and the A_1 layer are stained positively. Formal-calcium; Oil red O. ×210.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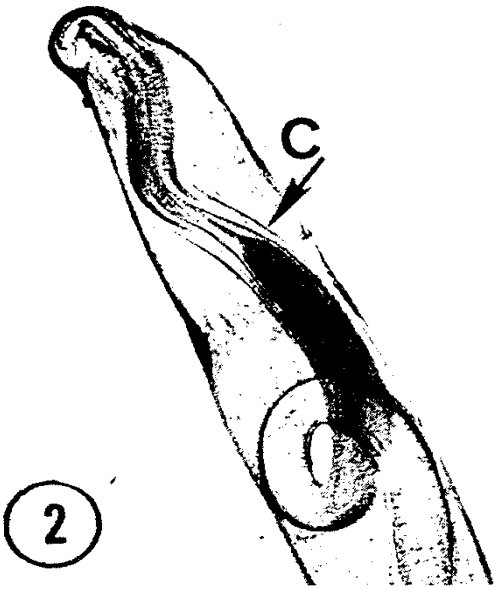
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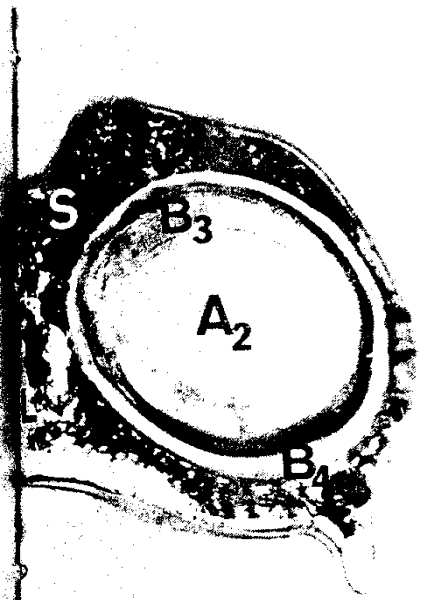
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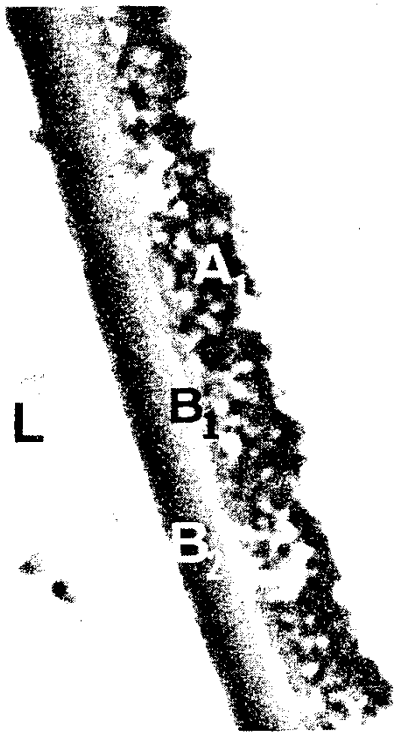
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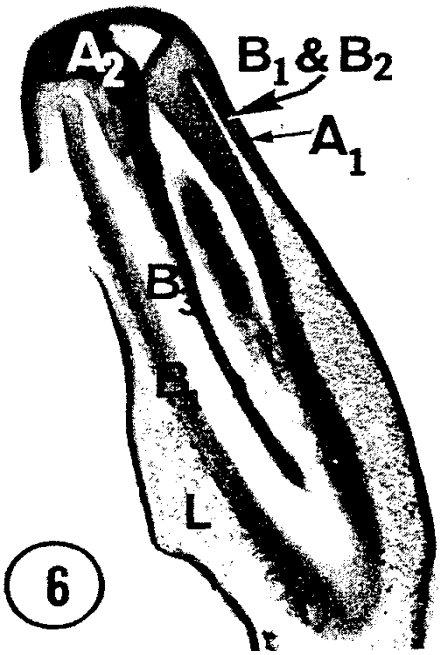
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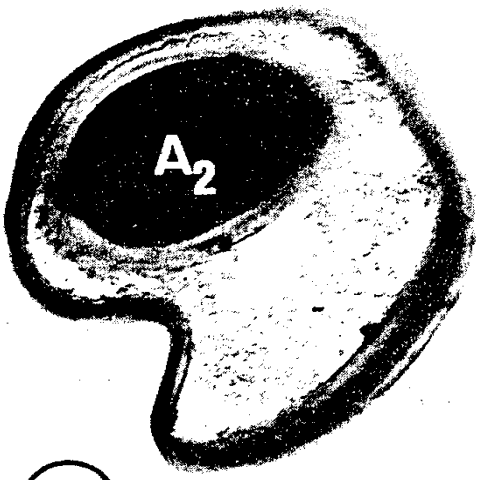
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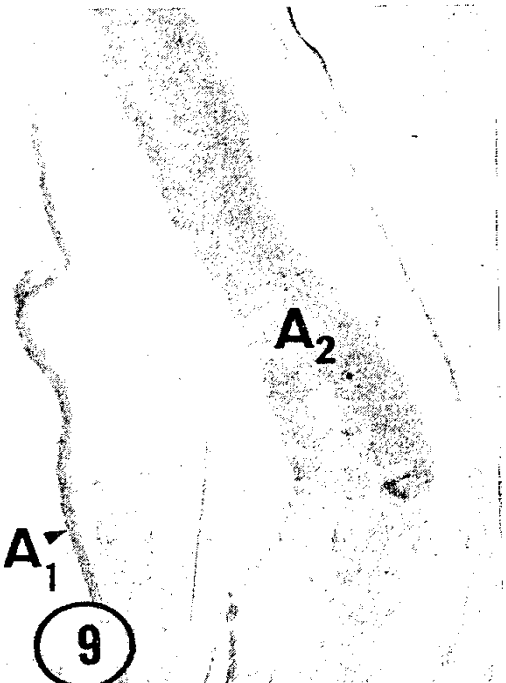
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phores were dehydrated in graded alcohols, propylene oxide, and embedded in Epon 812. Sections were cut on an OMU-2 ultramicrotome, retrieved on Formvar and carbon coated 200 mesh grids, and stained with methanolic uranyl acetate for 30 min followed by lead citrate for 5 min (Reynolds, 1963). Specimens were examined in an RCA EMU-2 E microscope at an accelerating voltage of 50 kV.

Observations and Results

The structure of the spermatophore

When a male and female *Tenebrio* are separated 25–30 sec after copulation has begun, the anterior tip of the spermatophore may be seen protruding from the male gonopore. At first the anterior portion of the sperm sac is opaque and the middle portion coiled. As the remainder of the sperm sac is expelled from the male, the coiling moves backwards to the translucent posterior portion of the spermatophore (Fig. 1). Generally a spermatophore retrieved from the male measured 3–5 mm in total length and 0·2 mm in diameter (Jones, 1967). Examination with phase contrast microscopy reveals that a multi-layered cylinder herein designated the *core*, is present in the anterior half of the sperm sac (Fig. 2). The core is narrow at the anterior, becomes wider in the middle, and then tapers towards its posterior tip. The core extends as far back as the first coil of the spermatophore in which region the tail of the core generally doubles upon itself (Fig. 3).

Fixed, sectioned, and stained material supports the results obtained with phase contrast. The general stain, Lower's trichrome (Lower, 1955) stains the spermatophore contents purplish blue (Fig. 4), while the outer and the middle layer of the core are stained deep red and the central substance of the core is only slightly stained. Results obtained with toluidine blue show that the tube wall also contains three layers (Fig. 5) and that the most external is frothy. For clarity, the frothy layers of the tube wall and of the core are designated as A_1 and A_2 respectively. The layer next to A_1 is designated B_1 and the innermost layer of the outer wall, B_2. In the core, the layer adjacent to the central material is designated B_3 and the innermost layer, B_4.

Fig. 3 shows a spermatophore fixed in osmium tetroxide; the central material (layer A_2) of the core is 'stained' black. Since osmium is known to react readily with lipid or lipoprotein deposits, this staining suggests that the central material may contain some lipid. Longitudinal paraffin sections (Figs. 6 and 7) and cross-sectioned material treated in sodium hypochlorite for 10 min (Fig. 8,

Table 1. *Staining reactions of the layers of the spermatophore*

	Tube wall layers		Core layers			
	A_1	B_1, B_2	A_2	B_3	B_4	Content
Heidenhain's iron-hematoxylin	+	+	+	+	+	+ + +
Lower's trichrome	+ + +	+ +	+	+ + +	+	+ + + +
Toluidine blue	+ +	+ + +	+ +	+ + + +	+	+
Millon's reagent	?	+	+ +	+ + + +	+	+
Alcoholic bromphenol blue	+ +	+ + + +	+ +	+ + + +	+ + +	+ + +
Dinitrofluorobenzene	+	+ + + +	+	+ + + +	+ + + +	+ + +
Dinitrofluorobenzene'						
$-SH$	+	+ + +	+	+ + + +	+ + +	+ +
$-NH_2$	0	+ + +	0	+ + + +	+ + +	+ +
Sudan black B	+ + + +	+ +	+ + + +	0	+ +	+
Oil red 0	+ + +	0	+ + +	0	0	0
Osmium tetroxide	?	+	+ + + +	+	+	+ +
PAS	+ +	0	+ + +	0	0	0
Azure A (pH 5·0)	0	0	0	0	0	+ +

0, Negative; +, weakly positive; + +, moderately positive; + + +, strongly positive; + + + +, intensely positive; ?, cannot be differentiated.

Wigglesworth's 1970 test for masked lipids) were all stained with Sudan black B. Frozen-sectioned material was also stained with Oil red 0 (Fig. 9). In all cases, layers A_1 and A_2 were deeply stained by lipid stains, while the B layers reacted only slightly.

All the layers and the contents of the spermatophore stain positively with the protein stains, bromphenol blue and DNFB (Fig. 15), but the A layers (particularly A_2) which gave the most positive reactions with lipid stains show relatively little protein. Both A_1 and A_2 stain very lightly with PAS; the B layers are unstained (Fig. 16). Except for the contents in the lumen, no layer of the spermatophore reacted positively to the alcian blue. Table 1 and the diagrammatic presentation in Fig. 10 summarize the staining reactions and the structural organization based on light microscopic results.

Electron micrographs considerably extend the picture obtained by histological and histochemical methods. In a cross-section of the anterior tip of the spermatophore (Fig. 11), the A layers of the tube wall and the core appear frothy with electron-transparent zones bounded by an electron-opaque framework. The homogeneity of the electron-opaque material is emphasized at higher magnification (Fig. 12). The electron dense framework of the A layer has approximately the same density and is continuous with the adjacent layers B_1 and B_3 (Figs. 11, 12, 14). The thickness of layer A_1 is variable and apparently there is no definite membrane at its external boundary (Fig. 13). An additional layer (C) forms an inner coat along B_2 and B_4 in most sections and although it is the same electron density as A_1 it appears rough and less organized than the latter (Figs. 13 and 14).

The lumen of a spermatophore freshly retrieved from a male is narrow at the anterior and contains sperm (Fig. 11). Posteriorly the

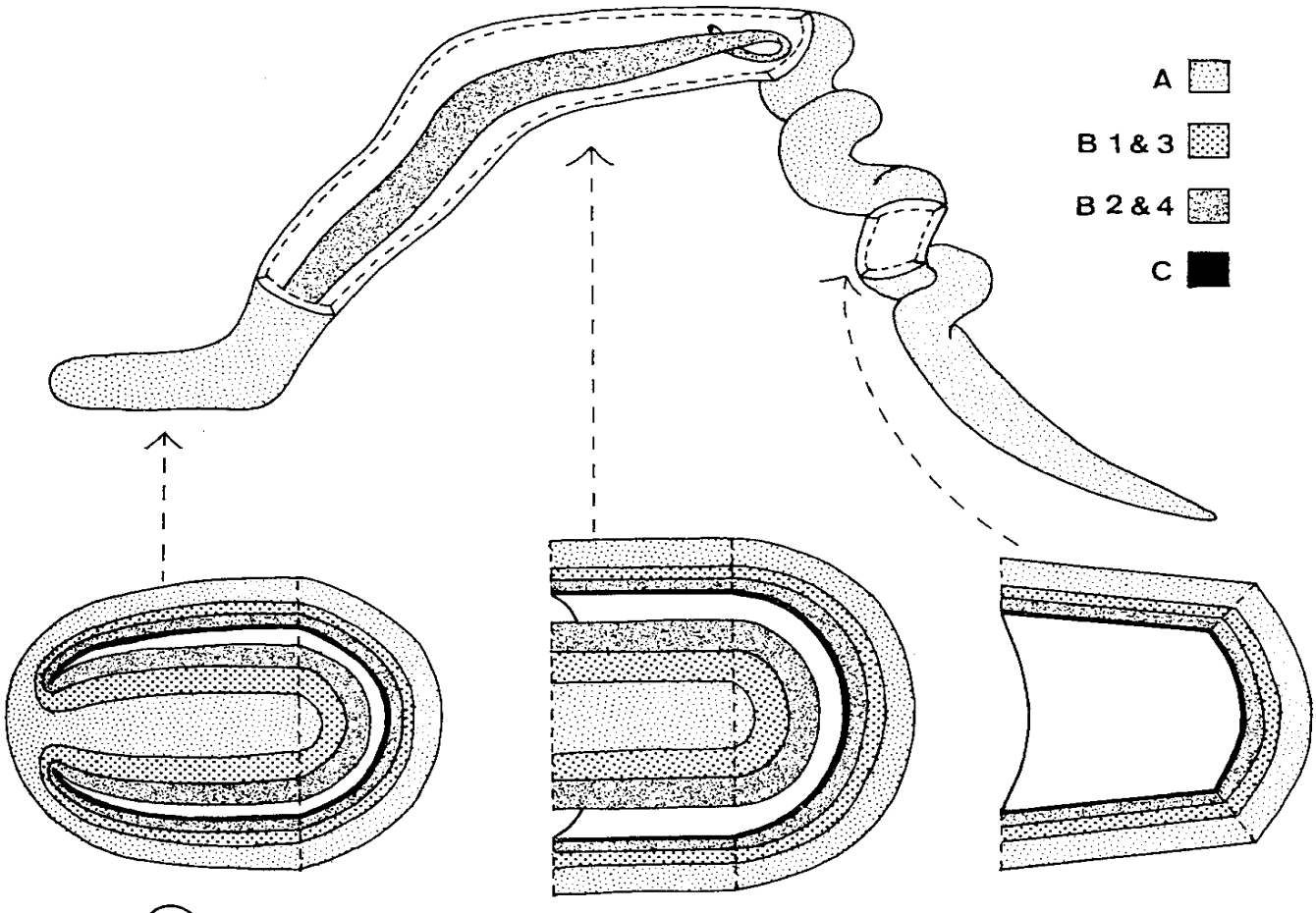

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Fig. 10. Diagrammatic illustration of the major structural features of the spermatophore retrieved from the male. The A_1, B_1, B_2, and C layers of the tube wall and the A_2, B_3, B_4 layers of the core are shown.

lumen widens and sperm are abundant (Figs. 13 and 14). Various collections of loosely organized product (P) are found in the lumen.

The evacuation of the spermatophore

Evacuation of a spermatophore involves four successive stereotyped stages. Within the female (or in saline) the spermatophore undergoes a two-stage elongation, then forms a bulb, and finally bursts (Figs. 18–23). The elongations are accompanied by changes in the distribution of the core content; it appears that the core everts itself at the most anterior tip. In the first stage (Fig. 19) the core moves forward, but some material (presumably from the A_2 layers of the core) persists at the site of the original tip of the spermatophore (Fig. 24). Phase contrast microscopy shows that, as the core moves forward, its central material A_2 is deposited along the external surface (Fig. 17). Four min after the spermatophore is placed in saline, a second elongation appears at a right angle to the first (Figs. 20 and 23) and the deposition of material at the base of the first elongation increases (Fig. 20). At this stage, the tail of the core has moved into the first elongation and the A_2 material protrudes out of the tip (Fig. 23). The formation of the bulb (effectively the third stage) follows the completion of the second elongation within one min (Fig. 21). Semen from the posterior region streams forward and fills the bulb. There is generally a jerk as though two structures are disconnected within the bulb about 30 sec after its formation. As shown in Fig. 25, the bulb is divided into two compartments which may be formed as a result of the separation of the layers B_3 and B_4. Sperm can be seen in the inner compartment before its collapse and apparently semen streams into the outer one (Fig. 26). When the bulb ruptures (about 1

Fig. 11. Electron micrograph of the spermatophore anterior. Layers A_1, A_2, B_1, B_3, and B_4 are readily distinguishable. The lumen (L) is narrow but contains sperm (Sp.) × 8800.

Fig. 12. The A_2 layer of the core at higher magnification. The electron dense material isolates electron transparent patches. These patches might be the original sites of lipid deposits which have been extracted during tissue preparation. × 44,000.

Fig. 13. Cross-section of the spermatophore showing A_1, B_1, B_2, and C layers of the tube wall. Some product (P) also lies along the (C) layer. Sperm (Sp) are present in the lumen. × 5200.

Fig. 14. Cross-section of the spermatophore showing A_2, B_2, and B_4 layers of the core. The B layers appear fibrous. Layer (C) is present along B_4. The lumen is large and contains sperm which are surrounded by product (P). × 8800.

Fig. 15. Spermatophore cross-section stained for protein. The B_3 and B_4 layers are most deeply stained. Bouin's; Dinitro-fluorobenzene. × 315.

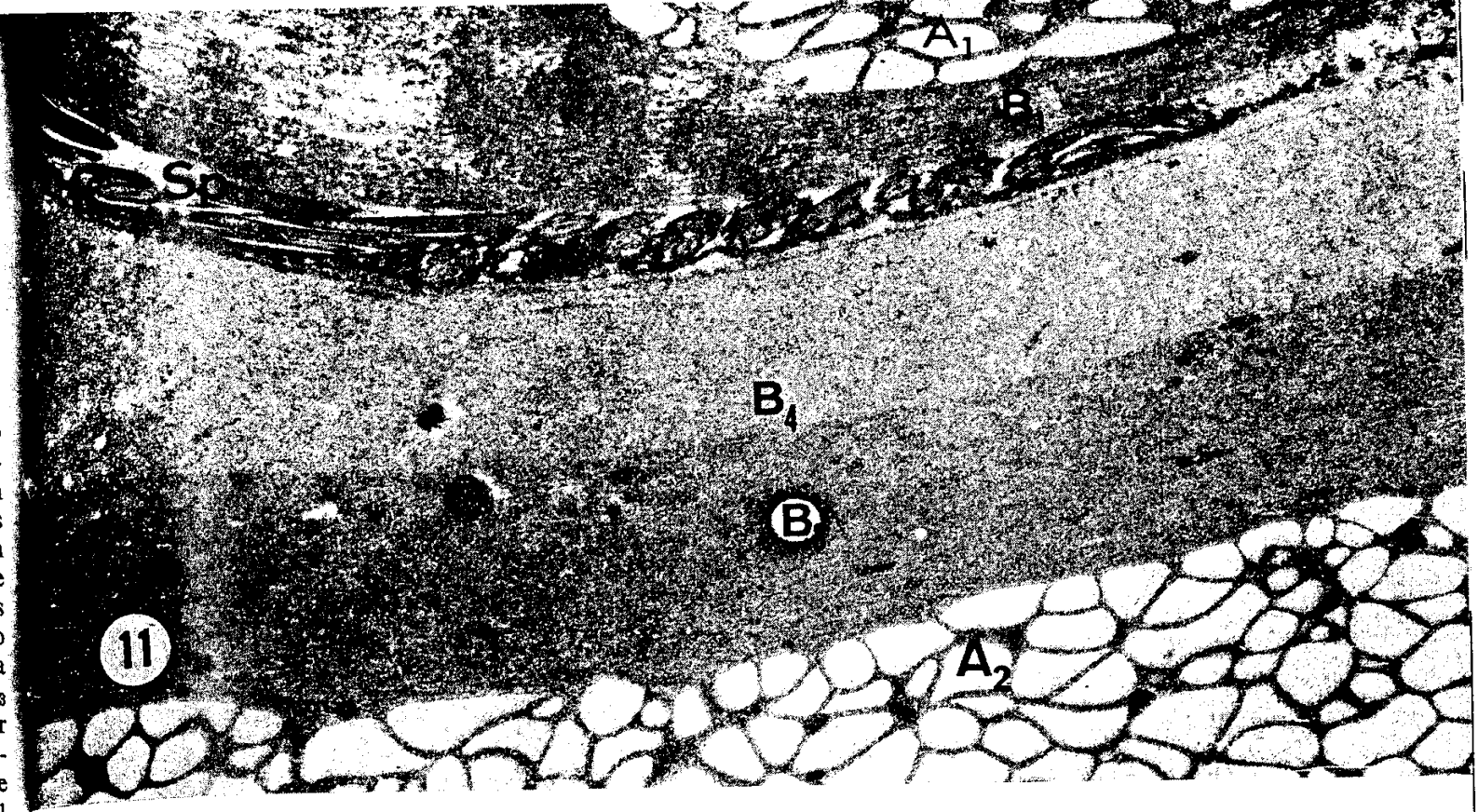
Fig. 16. Staining for the presence of carbohydrate in the spermatophore. Only the A layers of the sperm sac appear slightly stained. Bouin's; periodic acid Schiff. × 276.

Fig. 17. During the extension of the spermatophore the A_2 material of the core appears to be deposited on the external side of the tube wall. Phase contrast. × 360.

Figs. 18–22. Dark-field micrographs showing structural changes during the evacuation of the spermatophore. As sperm sacs extend, posterior portions become more transparent. See the text for further explanation. × 37.

Fig. 23. As the core everts during the formation of the second elongation, the tail of the core moves into the lumen of the first elongation. Note the A_2 material protrudes out at the anterior. Phase contrast. × 60.

Fig. 24. As the second elongation occurs, the deposit of the material (A_2) increases at the site of the original tip of the spermatophore. Phase contrast. × 300.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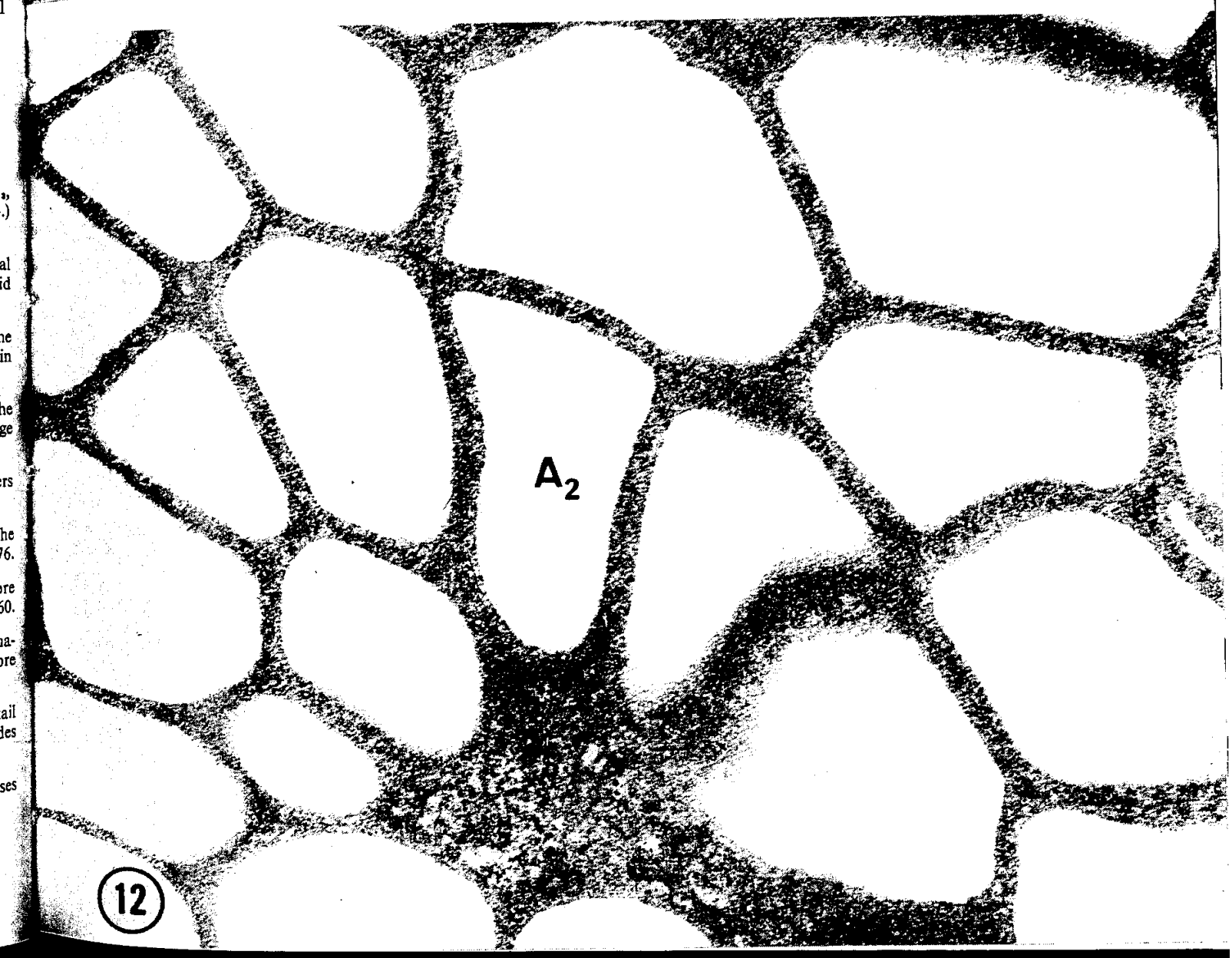
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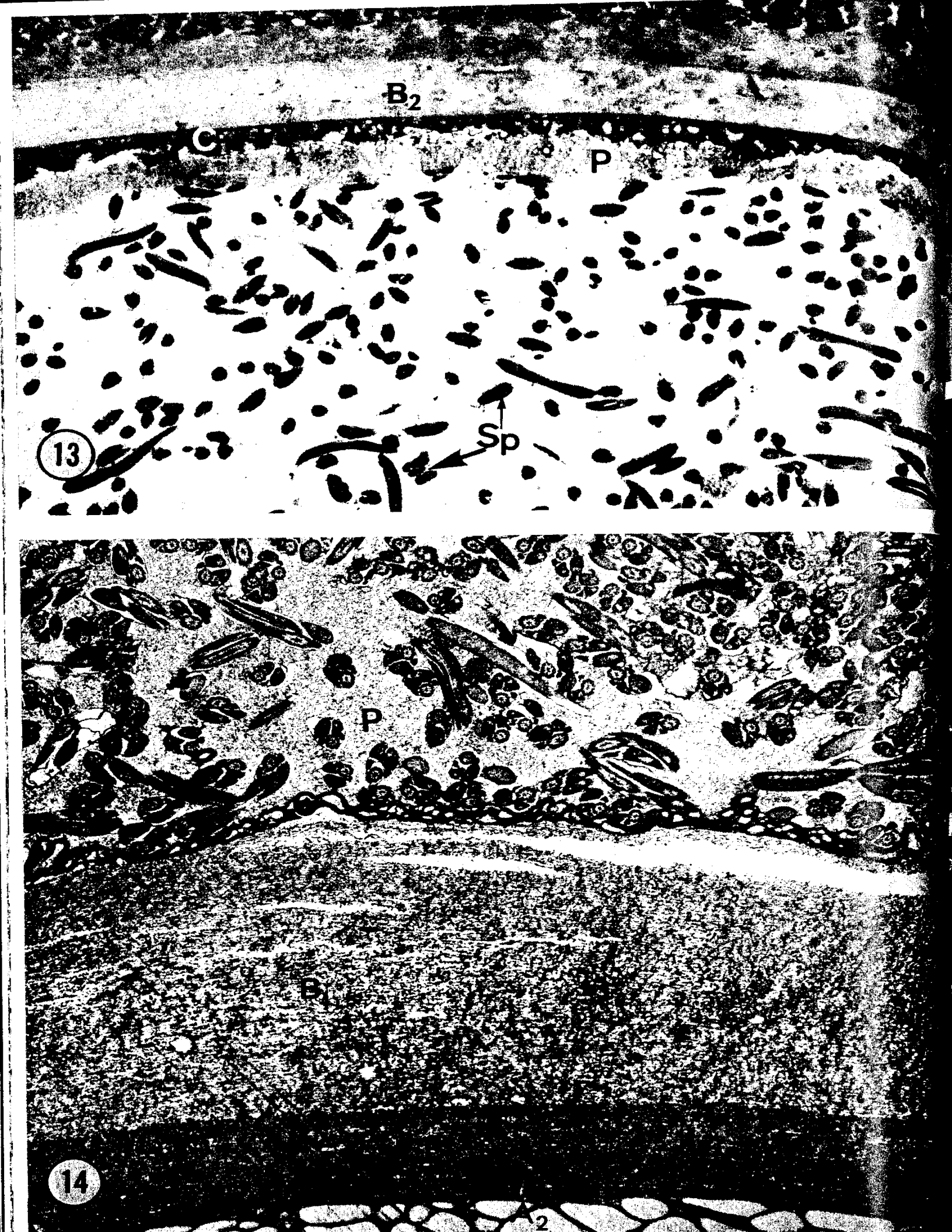
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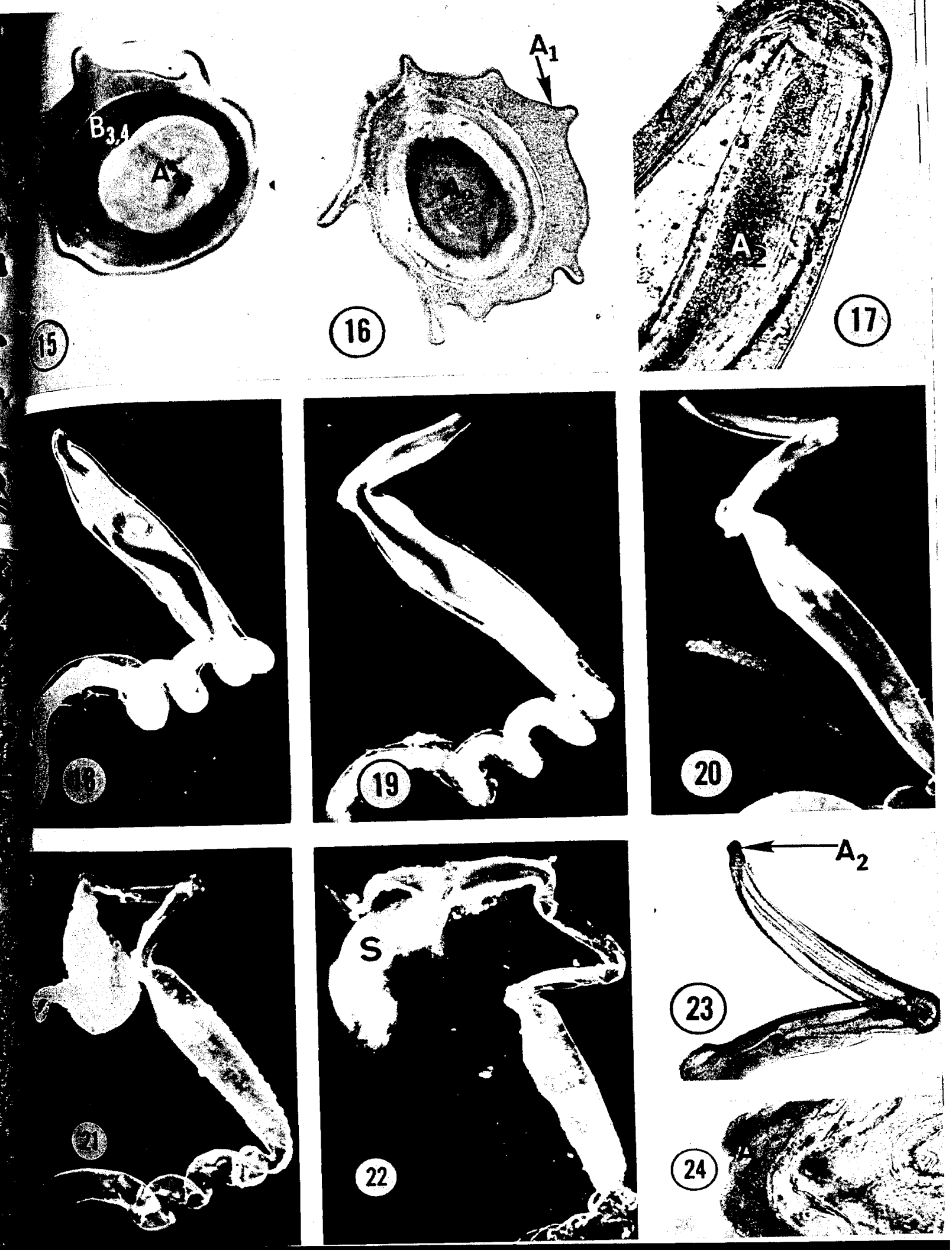
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min after its formation) the contents of the spermatophore are rapidly expelled with sufficient force to move the sperm sac out of the microscopical field. Upon evacuation, the bulb collapses.

Similar extensions occurred within the bursa of a female. Ten min after mating, all three stages had been completed and the bulb had collapsed (Figs. 27 and 28). When females were dissected in the second 5-min interval (5–10 min after copulation), the anterior tip of the spermatophore was often observed to project directly into the opening of the spermatheca.

Although sperm reach the smaller tubes of the spermatheca within 2 hr after copulation, the ruptured spermatophore (Fig. 30) is usually not ejected for about 18 hr. During this period, the A and B layers undergo some decomposition, but are still recognizable in ejected spermatophores (Figs. 35 and 36). Shortly after it has collapsed, the spermatophore is coiled tightly against itself, and while it lingers in the female bursa, the coiled sac becomes coated with an additional layer (designated D) which is apparently derived from the female. This coating stains bluish-green with Lower's trichrome (Figs. 31 and 32), is positive with PAS and alcian blue, has a slight affinity for DNFB and brom-

Fig. 25. The bulb (third stage) contains two compartments. The outer compartment (O Com) surrounds the inner one (I Com) in which semen (S) is present. Phase contrast. × 90.

Fig. 26. Sperm and other products are present in the outer compartment of the bulb before it bursts, releasing its contents. Phase contrast. × 93.

Fig. 27. Female bursa copulatrix (BC) containing a spermatophore (Spph). The anterior portion of the sperm sac, which is well beyond the junction of the common oviduct (CO) to the bursa, is in the proximity of the spermatheca (Spt). × 36

Fig. 28. Spermathecal opening (Spt) and the spermatophore (Spph) removed from a female 10 min after copulation. All the extensions have been completed. Formalin. × 36.

Fig. 29. Spermatophore retrieved from the bursa 30 min after copulation. The posterior portion of the spermatophore (Spph) is well wrapped in a translucent gelatinous material (D). × 36.

Fig. 30. Spermatophore, coated with gelatinous material, ejected out of female bursa 18 hr after copulation. × 120.

Fig. 31. Cross-section of the spermatophore (Spph) in the female bursa (BC) 40 min after copulation. A thin material (probably of protein–polysaccharide complex (D) partially wraps the spermatophore. Bouin's; Lower's trichrome. × 90.

Fig. 32. Cross-section of the female bursa (BC) containing a spermatophore (Spph) 4 hr after copulation. The wrap (D) appears thicker than in the previous figure. Bouin's; Lower's trichrome. × 90.

Fig. 33. Spermatophore ejected from the female stained for protein. The B layers are stained deeply and the gelatinous material (D) takes up some stain. Bouin's; bromphenol blue. × 120.

Fig. 34. Transverse section through spermatophore in the cuticle-lined (Cut) female. Even before ejection, layers A and B show signs of disintegration. Residual sperm (Sp) are still present in the lumen (L). × 8800.

Fig. 35. The horseshoe bent B layers of an ejected spermatophore appear fibrous on their disintegrating external boundaries. Small pieces of these layers appear distributed in the D layer. × 12,800.

Fig. 36. Electron micrograph of layers of spermatophore ejected from the female at higher magnification. The D layer (the gelatinous matrix) is seen to be composed of finely fibrous material. × 30,000.

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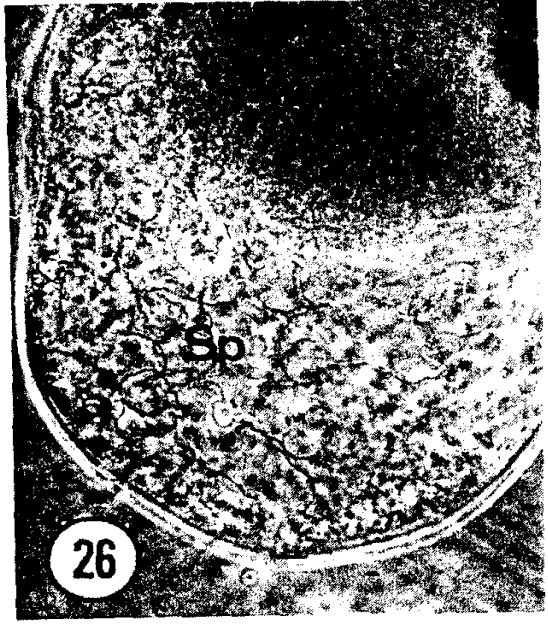

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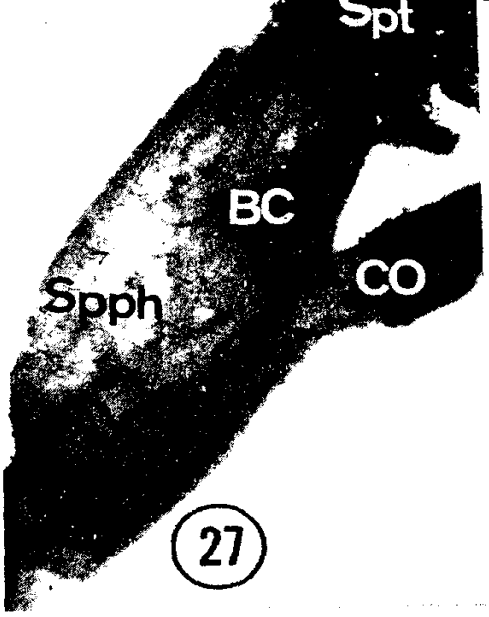

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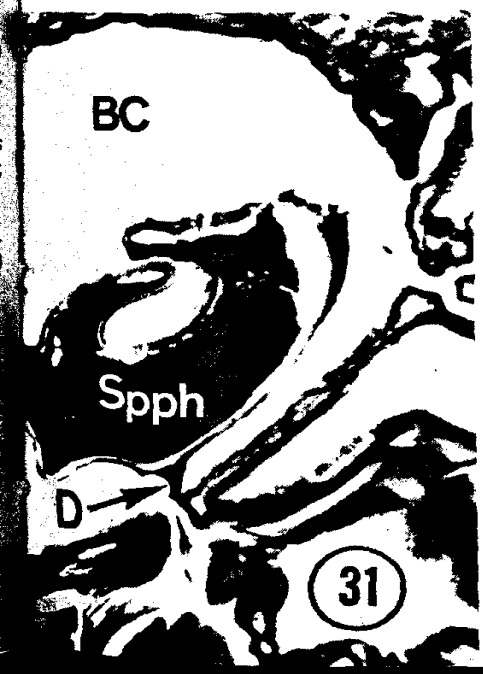

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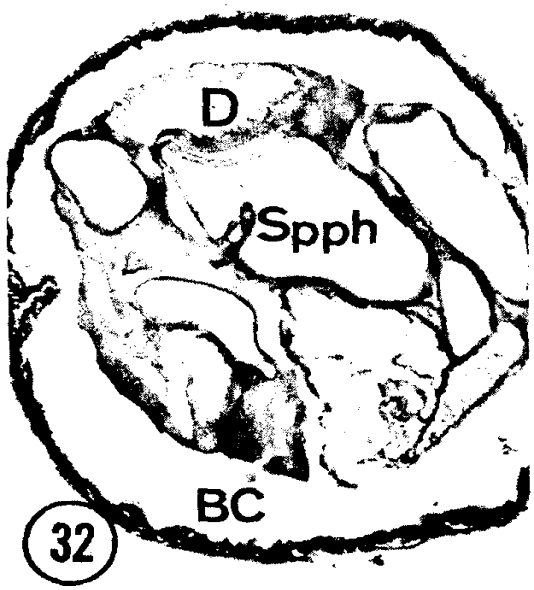

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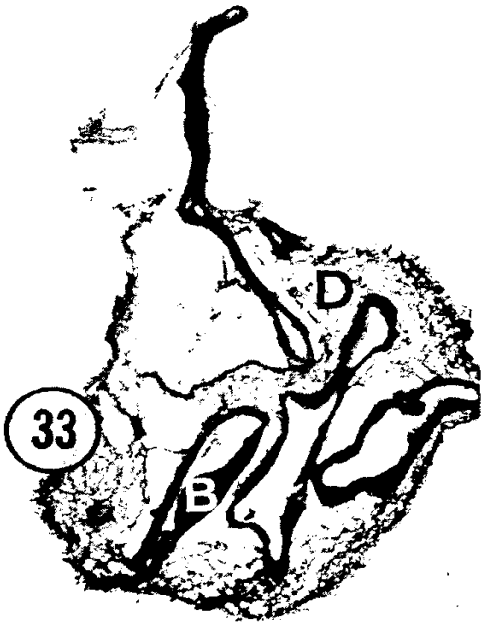

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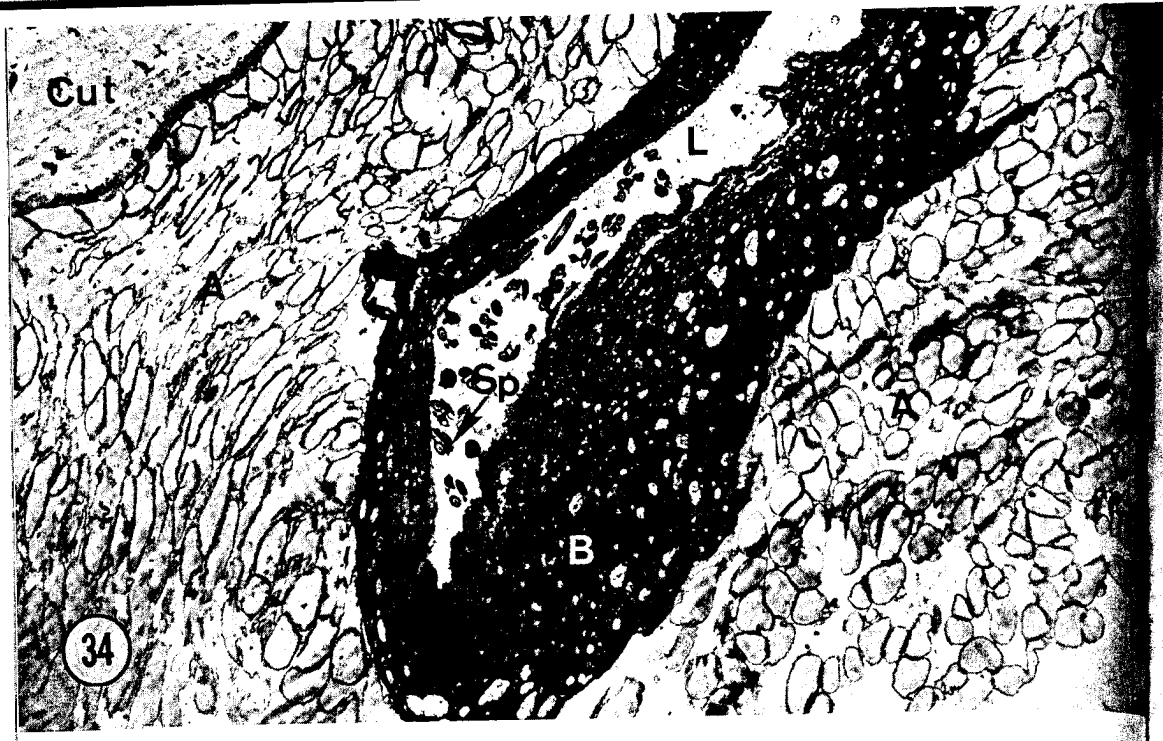

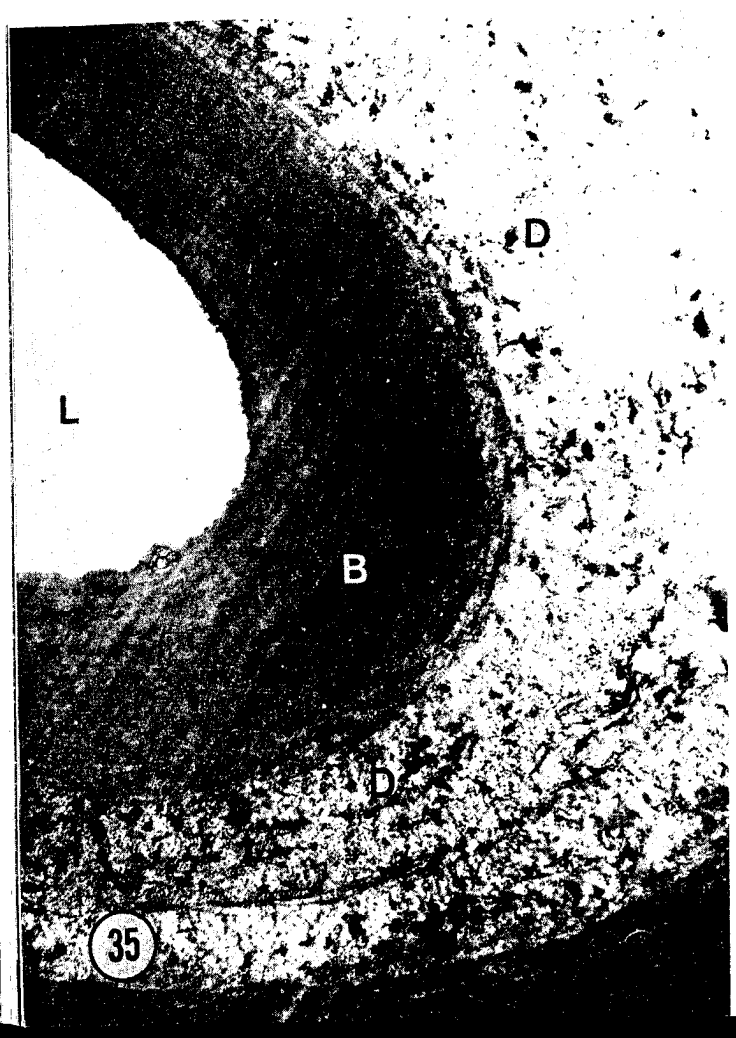

STRUCTURE AND

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phenol blue (Fig. 33) and appears fibrous in electron micrographs (Fig. 36). These characteristics are shared by the product of the accessory spermathecal gland (Happ and Happ, 1970) and presumably the coating is the secretion of this gland.

Discussion

As it emerges from the male ejaculatory duct, the spermatophore of *Tenebrio* consists of an outer tube and an inner core, both containing lipoprotein and protein. The wall of the tube is composed of an external lipoprotein layer covering two inner protein layers. The layers of the core are continuous with those of the wall, but with reversed orientation; the lipoprotein complex fills the centre of the core and is surrounded by two layers of protein. This arrangement is rather like a hollow blind tube, such as the finger of a glove, with its blind end invaginated to form the core.

A rapid and efficient transfer of sperm should be of adaptive advantage, since the duration of union (and relative vulnerability) is therefore minimized. In *Tenebrio*, copulation and sperm transfer are quite rapid—only 3–5 min. Since the sperm package is produced wholly within the male reproductive system and emerges fully organized within the first 30 sec of copulation, it must either be preformed in sexually competent males or assembled with great rapidity when females appear. And once within the female, the package ruptures.

The anterior end of the spermatophore expands in three stages within the bursa of the female. The third expansion, which forms a semen-filled bulb, is often placed at the spermathecal opening. To our knowledge, such a programmed sequence of expansions has not been described previously from other insect spermatophores. In a study of the spermatophore of another tenebrionid (*Pimelia angulata*), Fiori (1954) describes the long narrow coiled tube and an anterior bulb, but mentions no sequential extensions. It is possible that this spermatophore of *Pimelia* had undergone characteristic expansions before Fiori's observations were made.

The mechanisms which control the behavior of the organized acellular spermatophore of *Tenebrio* are not fully established. By analogy with other insects, osmotic pressure is a prime candidate. For example, Kalifa (1949a) concludes that sperm are expelled from the spermatophore of a cricket (*Gryllus domesticus*) when a 'pressure body' absorbs water, swells, and forces the sperm into a long narrow tube leading to the spermatheca of a female. In *Tenebrio*, the first elongation of the spermatophore is independent of the surrounding medium; it will occur even when a spermatophore is removed from a male and placed on a dry slide. However, the osmotic concentration of the surrounding media does influence the subsequent stages: either concentrated saline or dilute saline prolong their course. Furthermore, these stages proceed even after interruption. After 18 hr of desiccation on a dry slide exposed to room air, a spermatophore may still expand normally upon return to *Tenebrio* saline.

After the sperm mass is deposited in the female, the spermatozoa must be transferred to her spermatheca. At least three factors may affect this further transport of the sperm: strategic placement of the sperm mass, endogenous motility of the sperm, and peristaltic movements of the female tract. In some insects, notably the migratory locust (Gregory, 1965) and a blister beetle (Gerber, 1968), the spermatophore acts as an extension of the male copulatory tube to ensure proper placement of semen. In *Tenebrio* as shown in the present paper, the programmed behavior of the spermatophore itself plays a similar role even after male and female have separated. Most probably, peristalsis is also important. Davey (1958) has observed that rhythmic contractions of the vagina occur in mated female *Tenebrio*, but not in virgin females. Perhaps as in *Rhodnius* (Davey, 1958, 1965) secretions from the male initiate these contractions which then sweep sperm into the spermatheca. Endogenous motility of spermatozoa might assist their translocation, although, as Hinton (1964) points out, enthusiasm for this 'athletic prowess' hypothesis is diminishing.

For about 18 hr after it has ruptured, the spermatophore of *Tenebrio* remains in the bursa of the female, and during this time it becomes coated with secretions of the spermathecal accessory gland. What function does this coating serve? Although it might act as a lubricant during ejection of the spermato-

phore, we believe its presence on discarded spermatophores merely reflects contamination with female secretions. Apparently the muscular sphincter on the opening of the female accessory gland relaxes shortly after mating and the product diffuses into the bursa to be carried forward, by peristaltic contractions, past the ruptured spermatophore and finally to the spermatheca. *En route*, the secretions could sweep up spermatozoa which were free in the bursa. Both the persistence of the spermatophore and the flow of secretion maximize the number of sperm sequestered in the spermatheca.

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