THE EFFECT OF STRYCHNINE AND LIGHT ON PIGMENTATION IN *BLEPHARISMA UNDULANS* STEIN

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ABSTRACT

The effect of strychnine sulfate and light on pigmentation in the ciliate protozoan *Blepharisma undulans* has been determined. Upon exposure of cells to strychnine, the pigment granules become loosened from their surrounding membranes. Eventually these membranes break and the granules are simultaneously released from the cell. At the cell surface, a fusion occurs between adjacent membraneless granules with the incorporation of membrane fragments. This fusion of granules and membrane fragments results in the formation of a pigmented "capsule" around the organism. After elimination of the pigment, the granule membranes remaining in the cytoplasm fuse to form apparently empty vesicles. Other cell organelles are generally undisturbed. A similar situation occurs upon exposure of cells to artificial light for 12 to 18 hr, however, the slow elimination of granules from the cells under these conditions does not result in the formation of a pigmented "capsule." The possible mechanisms of these reactions are discussed.

INTRODUCTION

The reddish pigment characteristic of *Blepharisma undulans* is known to be influenced by a variety of physical and chemical factors. Thus, the organism is induced to shed its pigment when exposed to strychnine sulfate and certain other chemicals (15). The pigment layer is discarded as a unit, but the red stripes characteristic of the adult form are partially maintained in the pattern of the discarded material. A similar condition has been reported for *Stentor* (19). It has not been determined whether the discarded material represents the entire pellicle, a part of the pellicle as suggested by Nadler (15), or only extruded pigment granules normally located in the cortical region of the organism (11, 12). If the discarded material is a part of the pellicle or underlying cortex, it is difficult to understand how the process of shedding is brought about without injury to or disruption of the pellicular and cytoplasmic membranes. That is, how can a cell discard either its outer membrane or part of its cortical layer and immediately emerge with a functionally intact cell surface? One of the aims of this investigation has been to examine the detached "pellicle" in order to determine, if possible, its nature and origin.

After 8 to 12 hr of exposure to light, *Blepharisma* is completely bleached (7). A study of the fine structural changes associated with this process is also included.

MATERIALS AND METHODS

The organism used in this study was the ciliate protozoan *Blepharisma undulans* Stein (Strain NYU). Cells were grown in liter flasks containing 250 ml of

1% lettuce medium in Brandwein's solution (3) and were maintained in total darkness at pH 7.4 and at a temperature of 25°C. 12- to 14-day-old cultures of *Blepharisma*, in which the pigment granules were evenly distributed in the cortex just beneath the pellicle, were found to respond best to treatment with strychnine sulfate and light.

Ciliates were washed several times with Brandwein's solution and exposed to a 1/10,000 M solution of strychnine sulfate for varying time intervals (15). They were then fixed and prepared, as described below, for study with the light and electron microscopes. Pigmented "capsules," shed as a result of the strychnine treatment, were suspended on Formvar-coated grids, fixed in osmium tetroxide vapor, and studied as whole mounts with the electron microscope. Cells were also suspended in a 250-ml flask containing 50 ml of Brandwein's solution and exposed to a 60-watt light at a distance of 12 in. (final light intensity was 6 foot-candles) for 6, 12, and 18 hr. These cells were then prepared, as described below, for study with the electron microscope.

Cells for light microscope study were treated with the Chatton-Lwoff silver technique and Dragesco's (5) modification of Bodian's protargol technique. For electron microscopy, cells were fixed for 15 min in 1% osmium tetroxide containing a final sucrose concentration of 0.49% (buffered to pH 7.4 with 0.14 M veronal-acetate buffer). The organisms were dehydrated in rapid changes of ethanol followed by two changes of propylene oxide and embedded in Epon 812 (13) or Maraglas (6). Thin sections were cut with a Porter-Blum ultramicrotome, stained with lead hydroxide (4) or uranyl acetate, and examined with an RCA EMU 3D microscope.

OBSERVATIONS

Variation in the nature of the pellicle in ciliates has been responsible for a general absence from the the literature of a concise definition of this membrane. This situation has maintained for *Blepharisma* and other heterotrichous ciliates, primarily because of the difficulty in obtaining good fixation in this group. The pellicle in *Blepharisma undulans* does not appear as a unit membrane but rather as a single darkly staining layer when cut in cross-section and a lightly staining layer in tangential section (Figs. 6, 9, 10; 12, *PE*; and reference 12). A similar situation occurs with respect to the membrane around pigment granules. In both membranes, certain areas appear to be double membraned (Figs. 6; 12, *PE*), but this may be attributed to the plane of section and the irregularity of the membrane surface rather than to the existence of a unit membrane. This is unlike the situation in *Stentor* (17) in which the pellicle is composed of a distinct double membrane 400 A thick. It appears then that the outer surface of *Blepharisma* is a single membrane which serves as both a pellicle and plasma membrane. Whether a product of cell secretion is associated with this membrane, as suggested by light microscope studies (20), is not known. However, if this is the case, it may account for the dense staining features of the pellicle in this organism.

Pigmentation in *Blepharisma* is attributed to 0.4-μ membrane-bounded granules (Figs. 4, 9, *P*). In cell cultures harvested 12 to 14 days after inoculation, the pigment is distributed in rows of granules in the cell cortex (Fig. 9, *P*). Four to five distinct longitudinal rows of granules alternate with rows of cilia over the surface of the organism (Fig. 1, *PS*). Two types of granules have previously been described in the cortex (11, 12).

The Effect of Strychnine Sulfate

Various chemical agents induce the loss of pigment in *Blepharisma*. Primary among them is strychnine sulfate, used by Nadler (15) to produce "shedding" of the pigment which he concluded was confined to the pellicle. However, recent fine structure studies (11, 12) have demonstrated that the pigment is located in the cortex at the level of the kinetosomes rather than in the pellicle. Upon exposing 12- to 14-day cultures to a 1/10,000 M strychnine sulfate solution, a layer of pigment can be seen to loosen from the animal and form a "capsule" of material. The cell rotates within this pigment mass, emerges through the "capsule" in the oral region, and eventually swims away. The discarded "capsule" maintains its shape and pattern of pigment stripes (Figs. 2 and 3).

An analysis of this process has revealed that upon exposure of the cells to strychnine the pigment granules become freed from their surrounding membranes (Fig. 4, *P*). Eventually these membranes break, fuse with the pellicle, and simultaneously release the granules from the cell (Fig. 4). Fusion occurs among the ejected membraneless granules (Figs. 5, *CA*; 6, 7, *P*) and discarded membrane fragments (Figs. 5, *CA*; 7, *MF*). This fusion of granules and membrane fragments results in the formation of the pigmented "capsule" around the organism (Figs. 2 and 3; 5, *CA*). Following elimination of the pigment, the granule membranes remaining in the cytoplasm detach from the pellicle and some of them may

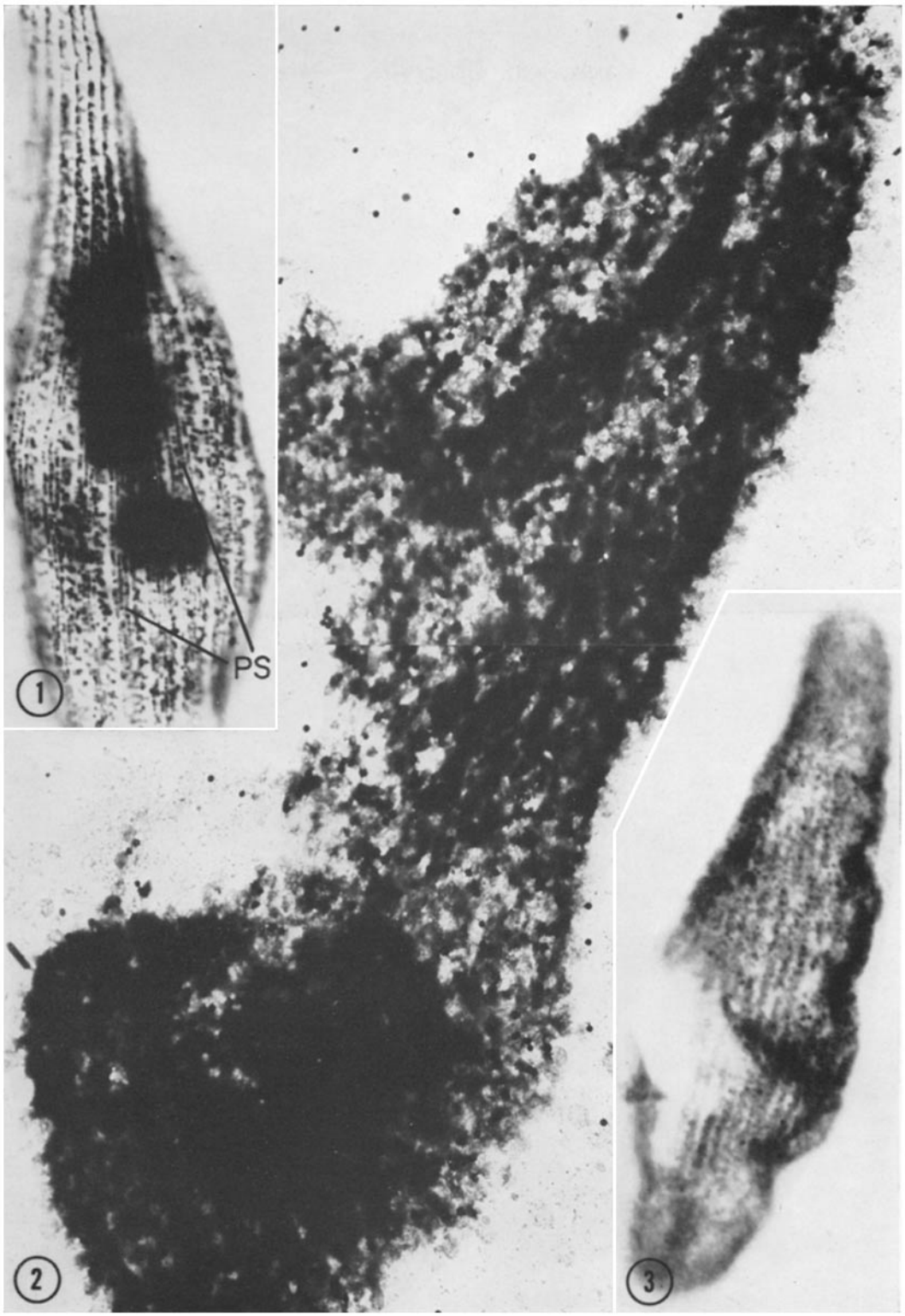
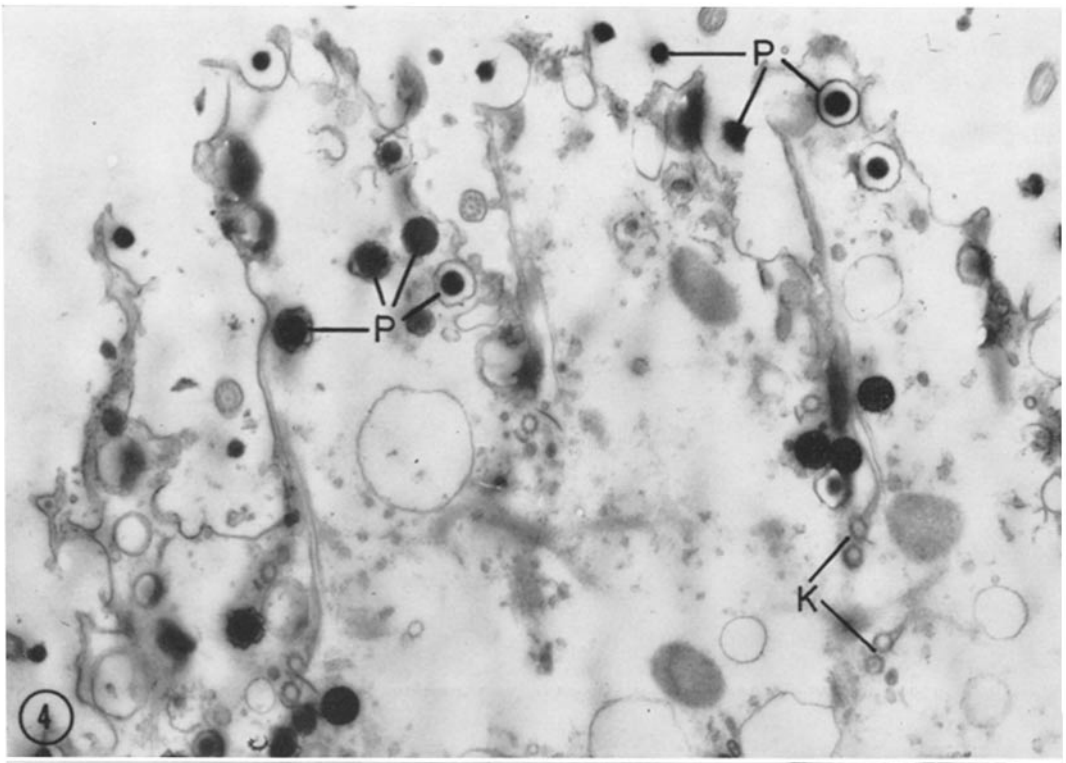


FIGURE 1 Light micrograph of a portion of a whole mount of *Blepharisma undulans* stained with protargol. The pigment stripes (*PS*) are composed of four to five rows of pigment. To the right of each stripe is a row of cilia. × 1,500.

FIGURE 2 Electron micrograph of a whole mount of the shed pigment "capsule" of *Blepharisma undulans* stained with uranyl acetate. In some regions the granular nature of this material is evident. × 3,100.

FIGURE 3 Light micrograph of a whole mount of a shed pigment "capsule" of *Blepharisma undulans*. Some indication of the pigment stripes is maintained. Stained with the Chatton-Lwoff silver technique. × 1,100.



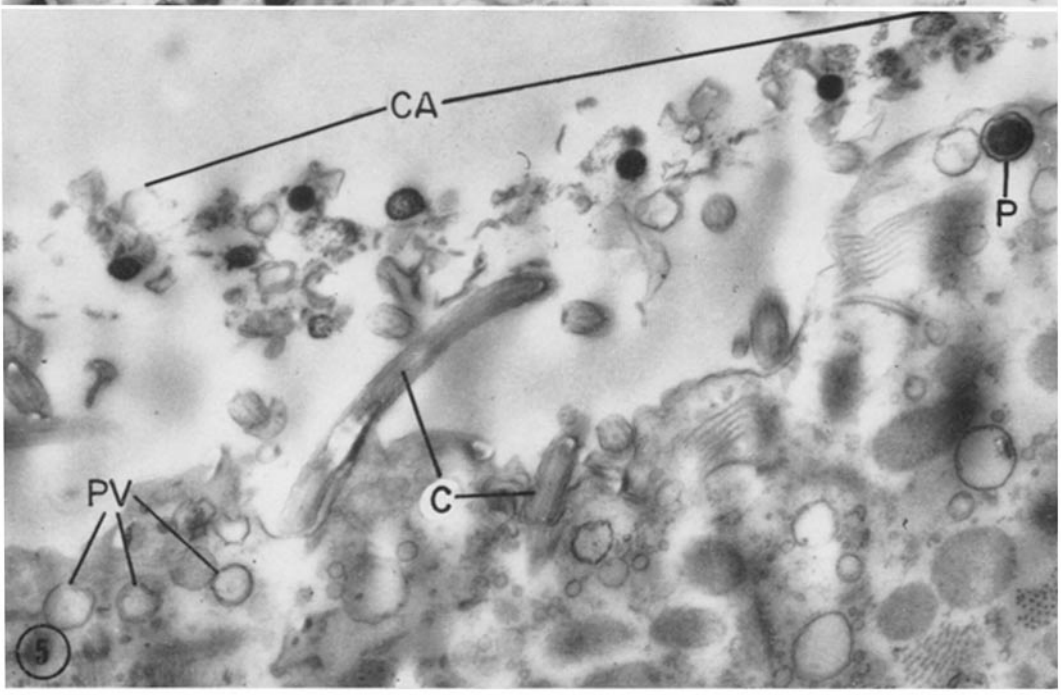


FIGURE 4 Electron micrograph of a tangential section through a *Blepharisma* cell exposed to strychnine. Pigment granules (*P*) are seen to loosen from the surrounding membrane. Some shrinkage of granules is evident. As the membranes rupture, the granules are discharged from the cell. The kinetosomes (*K*) with their cilia are apparently unaffected. × 12,500.

FIGURE 5 Electron micrograph of a longitudinal section through a portion of a *Blepharisma* cell and discarded "capsule." The "capsule" (*CA*) is composed of membrane fragments and pigment granules. Within the cell are an undischarged pigment granule (*P*) and vesicles devoid of pigment (*PV*). Extending from the surface are the cilia (*C*). × 17,000.

fuse to form empty vesicles in the region of the cytoplasm formerly occupied by pigment granules (Figs. 5, 8, *PV*). The pellicle soon returns to a morphologically normal condition. Other cell organelles, such as the cilia (Fig. 5, *C*) and kinetosomes (Figs. 4, 8, *K*), are generally undisturbed and cell motility persists. However, exposure of these depigmented cells to high intensities of light results in their immediate lysis. Nadler (15) reports that the pigment can be regenerated if cells are transferred to fresh media.

The Effect of Artificial Light

Morphologically, the effect of light on pigmentation is similar to that of strychnine, requiring only a longer time for completion. Exposing cells to a light intensity of 6 foot-candles for 6 hr causes no apparent reduction in cell pigmentation (Fig. 9, *P*); however, after 12 hr of exposure there is considerable loss of pigment. Fine structure examination reveals that the membranes of the granules rupture and that pigment granules are ejected from the cell (Figs. 10, 11, *P* and arrow). Following elimination of the granules, the remaining granule membranes fuse, as in strychnine-treated cells, to form the characteristic vesicles devoid of pigment (Figs. 10, 11, *PV*). Further exposure to light (18 hr) only enhances the effect, resulting in nearly complete depigmentation of the organism (Fig. 12, *P*). While membrane fragmentation has been observed in light-treated animals, it appears to be of much lesser degree than in strychnine-treated ones. This may be attributed to the long time-period required for total discharge of pigment. The absence of large accumulations of pigment granules and membrane fragments on the cell surface at a given time may account for the failure of "capsule" formation in light-treated cells. Upon release of the pigment granule, the membrane that previously surrounded it fuses with the pellicle (Fig. 12, *PE*). However, this membrane is not incorporated into the pellicle, but presumably forms the vesicle as seen in Figs. 8 and 11 (*PV*). Shrinking of the pigment granules as observed in the strychnine-treated cells (Fig. 4) is not so apparent in cells exposed to light (Figs. 10, 11).

DISCUSSION

Two ciliate genera with which pigmentation is commonly associated are *Blepharisma* and *Stentor* (for a review of the properties of pigments of these and other ciliates, *see* reference 9). Since Arcichovskij (1) first named the pigment in *Blepharisma* zoopurpurin, considerable information has been accumulated concerning its physical and chemical properties. However, little is known about its composition. Giese (9) reports that it is not a large molecule, and Prabhakara Rao (16) indicates it to be associated with protein in its natural state. Extensive studies on the nature of stentorin (14), the pigment of *Stentor*, have demonstrated that this pigment, also granular, may be combined with carbohydrate, nucleic acids, or a special group of proteins. According to Møller (14), both stentorin and zoopurpurin probably belong to the meso-naphtho-dianthrone group of compounds. This group also includes the photodynamic pigments hypericin and phagopyrin, and has thus far been described only for plants. A recent study by Servenants (18) confirms the similarity of zoopurpurin to hypericin, with the differences arising primarily in the position and number of functional groups.

A variety of functions has been proposed for zoopurpurin (for a review, *see* reference 14). The most popular suggestion has been that the pigment offers protection against predation. Giese (8) reports that upon exposure to a brei of *Blepharisma* fragments *Paramecium* reacts violently and quickly dies. That pigment would serve as a general protective agent seems unlikely, though, since Giese further observed that *Blepharisma* can be fed to a variety of protozoa without producing toxic effects. Møller (14) states, "the most attractive hypothesis seems to be that these pigments mediate the orientation of the organism in a light gradient." More information will be required before a definite function can be attributed to the pigment.

The Effects of Strychnine and Light

The process of pigment shedding in *Blepharisma*, first reported by Nadler (15) to occur following exposure to strychnine sulfate, cocaine hydrochloride, codeine sulfate, and novocain, has now been produced with other agents including Janus green (21) and a series of salts (16). A similar process, also induced by these compounds, has been described in *Stentor* (19). Rather than the shedding of the pellicle, as described by Nadler, the process involves rupturing of membranes and elimination of the pigment as described above. Prabhakara Rao (16) proposed "that the removal

of calcium from the outer membrane by chemicals disintegrates the pellicle and produces the extrusion." It should be noted that both pellicle and granule membranes rupture only where pigment granules are associated with the pellicle. Further, the ruptured pellicular membranes subsequently fuse to return the pellicle to its former structural state. This fusion occurs in the presence of the agents which originally induced rupture of the membranes. The suggestion, in certain micrographs, of structural differences between pellicular and granule membranes is not consistent or dependable. The pigment granule membranes and the pellicle appear to be somewhat compatible, as indicated by the fusion of the two membrane systems at pigment discharge. However, this compatibility is only temporary since upon discharge of the pigment the pellicle returns to its former structural state, as does the pigment granule membrane. Thus a membrane-bounded pigmentless vesicle is seen in the cortex of colorless animals. The pellicle, while normal in appearance, may have lost much of its former stability since cells induced to shed their pigment show increased sensitivity to pressure (2). Those cells in which the pigment is not closely associated with the pellicle do not show any short-range effects of the strychnine, such as shedding of pigment. In addition, studies on the effect of strychnine on the spinal cord (22) indicate a dilation of the spaces of the endoplasmic reticulum of the neurons, but no membrane damage is reported. Thus, it seems unlikely that strychnine acts directly on the membrane or a component of the membrane.

The action of light on pigment has been known almost as long as the effect of strychnine (7). Present evidence indicates that the effects of these two agents are similar, although the mechanisms underlying the effects may be different. Thus light also induces the elimination of pigment granules from the cell, but over a longer period of time. The question arises as to how the breakage of membranes is produced; and in both cases, two general mechanisms must be considered. First, as Prabhakara Rao (16) has proposed, the agents may be affecting the membranes directly. This seems unlikely, particularly in the case of the effect of light on the pigment. Secondly, the physical and chemical agents involved may be reacting with the pigment directly, bringing about a subsequent rupture of the membranes and liberation of the granules. Photo-oxidation of pigment (10) in cells subjected to light may cause such a reaction.

In the absence of further information, it may be suggested that rupture of the membranes in strychnine- and light-treated animals results from a reaction of these two agents with the pigment, causing a subsequent breakdown of the membranes, rather than directly affecting the membranes themselves.

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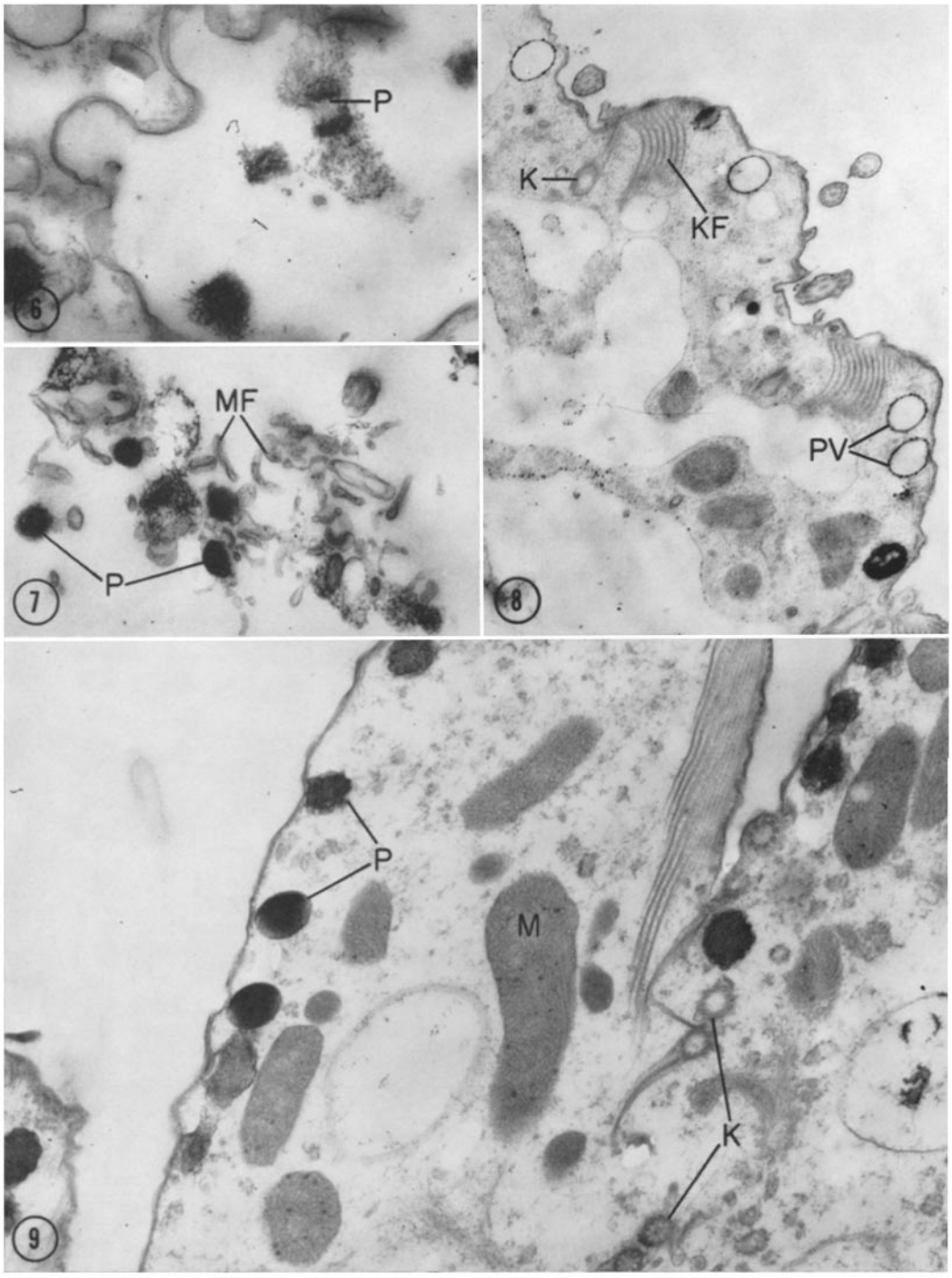
FIGURE 6 Electron micrograph of the surface of a *Blepharisma* cell treated with strychnine. The pigment granules are being discharged from the cell. Two of these granules are apparently in the process of fusion (*P*). × 31,000.

FIGURE 7 Electron micrograph of a section through a portion of the discarded "capsule" of *Blepharisma*, showing that the "capsule" is composed of pigment granules (*P*) and membrane fragments (*MF*). × 28,500.

FIGURE 8 Electron micrograph of a portion of the *Blepharisma* cell surface after strychnine treatment. Following discharge of the pigment, the granule membranes fuse to form apparently empty vesicles (*PV*). Kinetosomes (*K*) and kinetodesmal fibers (*KF*) are unaffected. × 19,000.

FIGURE 9 Electron micrograph of a longitudinal section through a *Blepharisma* cell exposed to light for 6 hr. Little bleaching has occurred. Pigment granules (*P*) are arranged in rows just beneath the cell surface. Kinetosomes (*K*) and mitochondria (*M*) are also evident. × 25,000.


P
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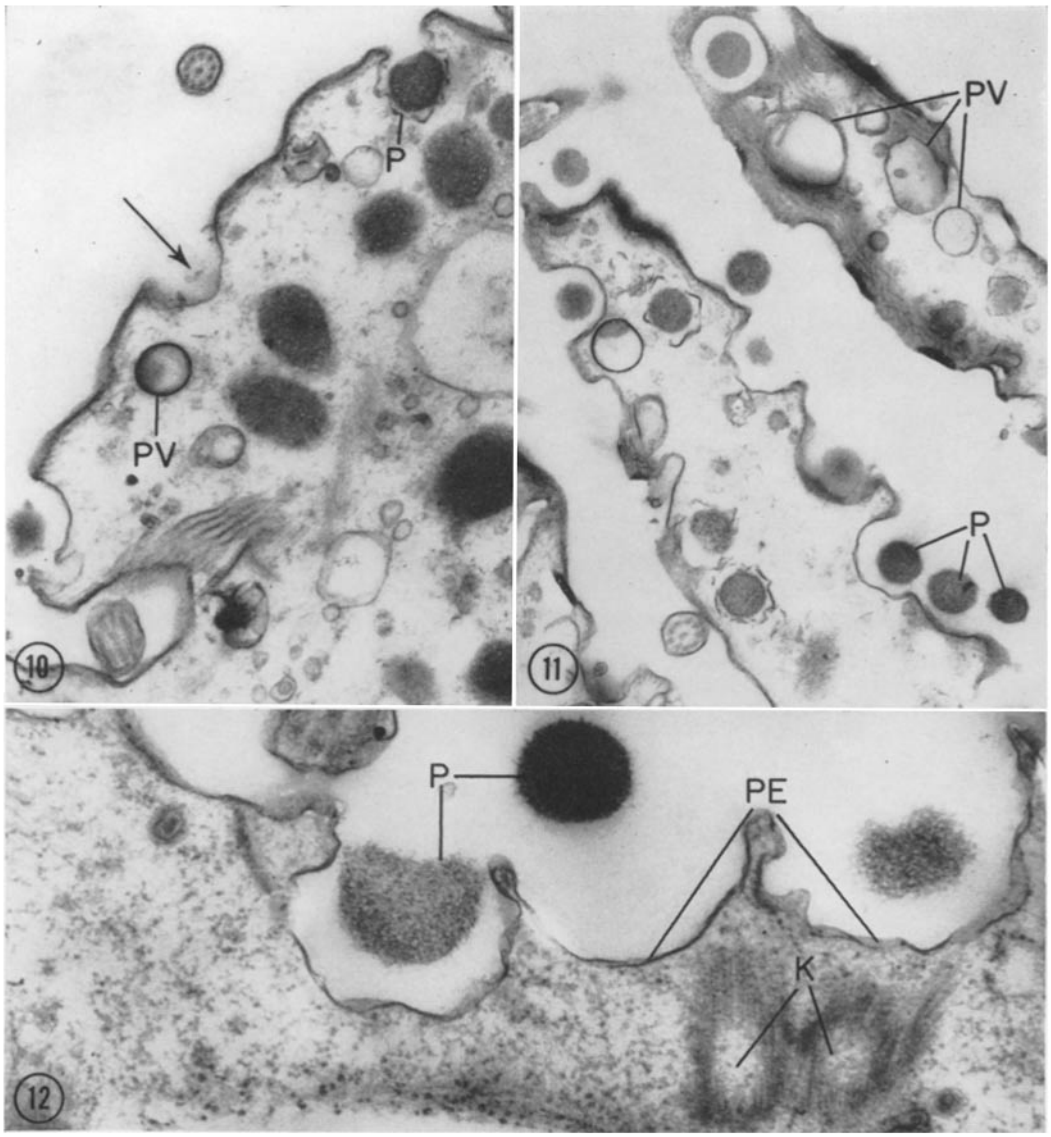

FIGURE 10 Electron micrograph of a portion of a *Blepharisma* cell exposed to light for 12 hr. The ruptured membrane of pigment granule (*P*) has fused with the pellicle, and the pigment is about to be discharged from the cell. Following discharge of the pigment, an empty depression (arrow) remains in the cell surface. Fusion of the granule membranes then takes place to form a pigmentless vesicle (*PV*). × 19,000.

FIGURE 11 Electron micrograph of the surface section of a *Blepharisma* cell after 12 hr of light exposure, showing pigment granules (*P*) that have been ejected and pigmentless vesicles (*PV*). × 18,000.

FIGURE 12 Electron micrograph of *Blepharisma* cell after 18 hr of light exposure, showing that discharge of pigment granules (*P*) is still occurring but nearly complete. The pigment granule membranes appear continuous with the pellicle (*PE*), and the kinetosomes (*K*) are undisturbed. × 62,000.

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