# An Optional Dyadic Junctional Complex Revealed by Fast-Freeze Fixation in the Bioluminescent System of the Scale Worm

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*Abstract.* In the bioluminescent system of the scale worm, the facilitation of the successive flashes is correlated with the progressive recruitment, in each photogenic cell, of new units of activity, the photosomes. To characterize morphologically the coupled state of the photosomes, known to decouple within seconds at rest, fast-freeze fixation was applied to stimulated and nonstimulated elytra and followed by substitution with $OsO_4$ in acetone. The results showed striking differences. Photosomes were surrounded by a new type of smooth endoplasmic reticulum (ER) called intermediate endoplasmic reticulum (IER). In nonstimulated elytra, the IER was most often unattached in the cytoplasm. After stimulation, the IER was connected to large terminal saccules that formed dyad junctions with the plasma membrane. Most of these junctional complexes were symmetrical (triads) and occurred in front of narrow extracellular spaces. These spaces were either constitutive, like invaginations or clefts along adjacent cells and adjacent pouches, or resulted from the pairing of long pseudopods which expanded into a wide extracellular compartment and twisted together in a dynamic process. In that the junctional complexes developed progressively under repeated stimulation and coupled more and more photosomes, they must represent a route constituted by the ER for the propagation of internal conduction. The dynamics of coupling involve membrane growth, recognition, and transformation on a surprisingly large scale and in a surprisingly short time.

O UR previous study demonstrated the existence of an intracellular mechanism of recruitment that correlated with facilitating responses in the bioluminescent system of the scale worm (4). It was thus postulated that photosomes, the intracellular units of activity, are either coupled and able to flash by stimulating the photogenic cell, or uncoupled and unreactive. It was established that the coupled state has a limited lifetime on the order of 1 s, that this basic duration can be reinforced under sustained stimulation, and that the constructive action by which uncoupled photosomes become coupled during the recruitment process is a function of the frequency of stimulation. We also postulated that some photosomes must be precoupled, in order to be ready to respond to the first stimulation.

Coupling thus appears as an optional switch-in device localized at the level of individual photosomes, and as an absolute requirement for them to react to stimulation. What is the morphologic basis of the coupled state? Functionally, how does the coupling mechanism link excitation and bio-

luminescence, and what are the dynamics of its construction and destruction?

Photosomes are paracrystals of tubular endoplasmic reticulum (ER)[1] (1). Coiled ER tubules arranged in parallel rows, which are themselves repeated in parallel or intercrossing planes, develop a considerable membrane surface that separates an intracisternal compartment (i.e., the lumen of the tubules) from the cytosol. The membrane bears polynoidin, a photoprotein which in isolated form emits light when triggered by oxygen radicals (25, 26). These radicals, particularly the superoxide ion, might be produced by the oxidation of the reduced flavin present in the photosomes, which accounts for the observed development of fluorescence correlated with bioluminescent activity (2–7).

Electrophysiological studies by Herrera (19) and Bilbaut (8, 9) showed that the photogenic epithelium, whose cells are linked by gap junctions, propagates an $Na^+$ $Ca^{2+}$ action potential. It was concluded from experiments with different ionic media that the $Ca^{2+}$ influx triggers the luminous reaction. Nevertheless, neither the facilitation nor its underlying coupling mechanism was considered in these studies. Additionally, the fact that polynoidin luminescence is not triggered by $Ca^{2+}$ in vitro (26) suggests that the intracellular part of the excitation–bioluminescence pathway might involve a more complex mechanism than just diffusion of a triggering ion as in the case of coelenterate photocytes (13).

The first structural evidence that a differentiated junctional

1. *Abbreviations used in this paper:* DER, dyadic endoplasmic reticulum; ER, endoplasmic reticulum; FFFS, fast-freeze fixation followed by freeze substitution; IER, intermediate endoplasmic reticulum; PER, paracrystalline endoplasmic reticulum.
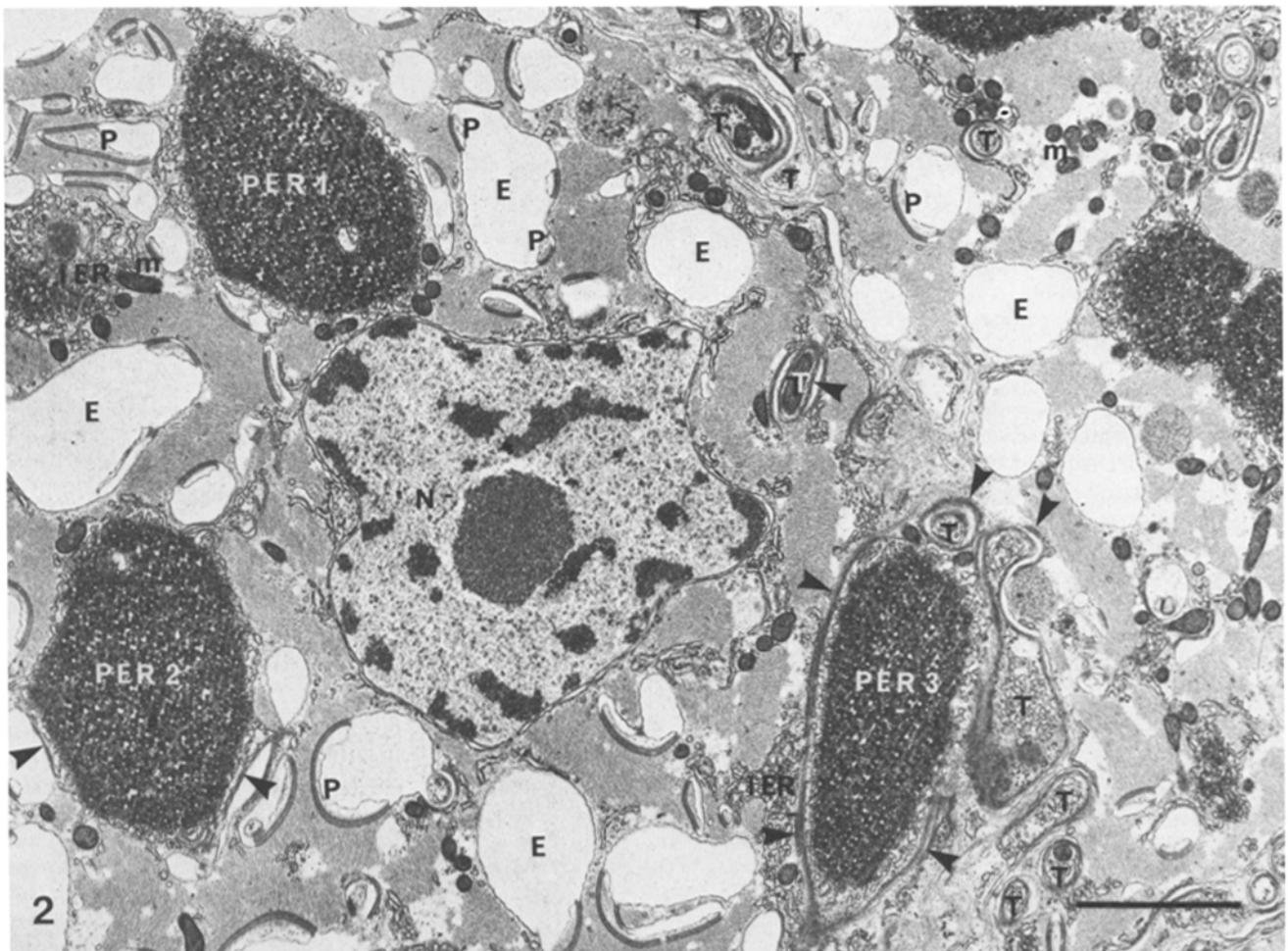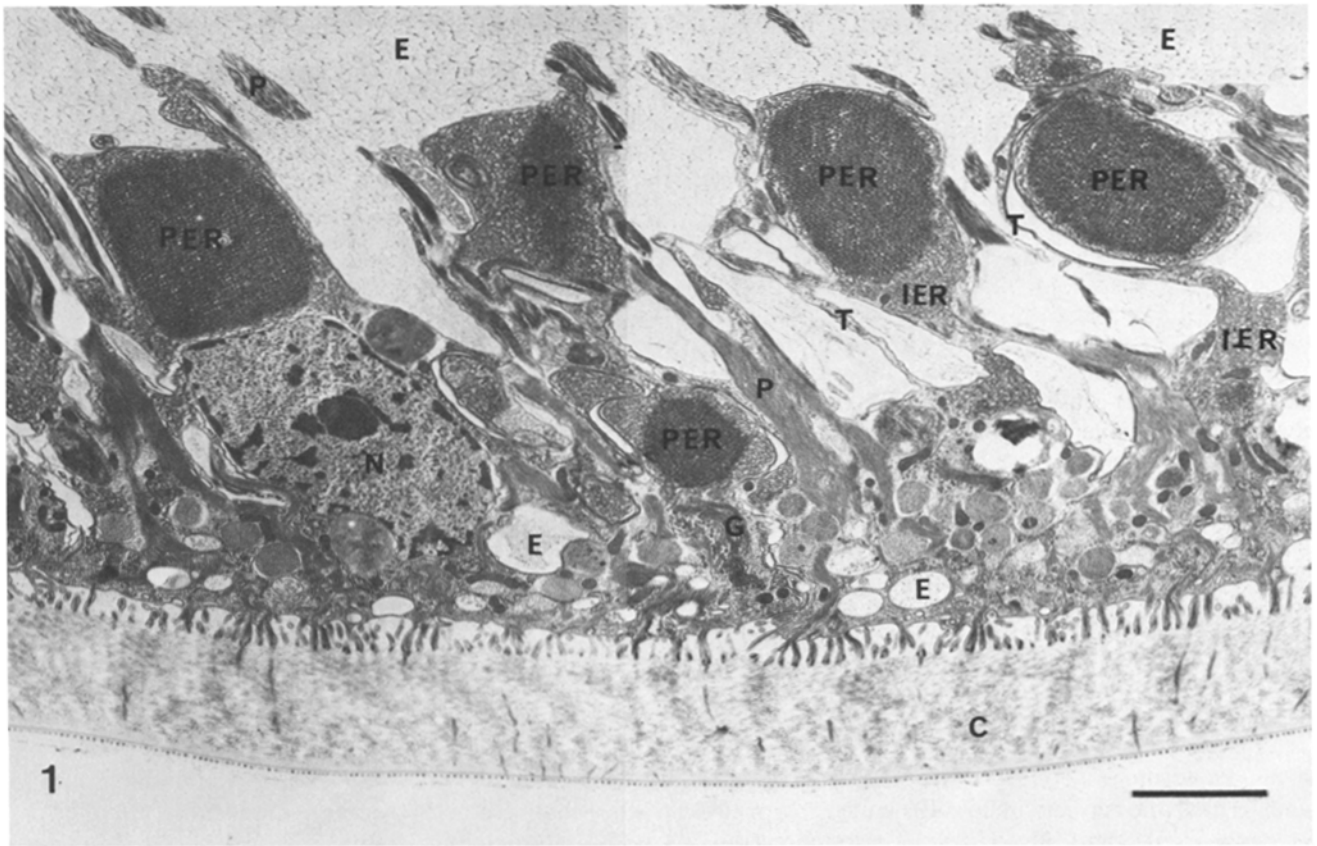
complex associates individual photosomes with the plasma membrane was given by Pavans de Ceccatty et al. (31, 32). Very occasionally, photosomes were observed to be directly connected to large, flat saccules of ER running parallel to the plasma membrane. These junctions were named dyad junctions because they resembled those of the muscle system and were thought to constitute an electrotonic coupling by which the excitation could reach a photosome. Assuming that the rarity of this observation was due to the fact that the lifetime of the coupled state was too short to be securely captured by usual chemical fixation methods, which penetrate slowly, we tried to improve the rapidity of the glutaraldehyde fixation with quick, standardized manipulations at precise stages of the bioluminescent activity. With this procedure, stimulated elytra always exhibited a large number of dyad junctions.

A decisive improvement was brought about by a fast-freeze fixation technique, followed by freeze substitution (FFFS) (18, 21, 37, 38). The thin, flat photogenic area of the scale-worm elytra proved to be a particularly suitable material for this procedure (3). In many instances, the general cytologic preservation was considerably improved compared with that obtained after conventional chemical fixation. It led to new observations, especially those concerning the astonishing plasticity of the cell wall which faces the middle compartment of the elytrum and those concerning a hitherto unknown part of the ER, which we called the intermediate endoplasmic reticulum (IER). In stimulated elytra, this IER establishes a link between the paracrystalline endoplasmic reticulum (PER) of the photosomes and the terminal saccules of the dyadic endoplasmic reticulum (DER).

## Materials and Methods

All experiments were performed on the scale worm *Harmothoe lunulata* (annelids Polychaetes, Aphroditidae, Polynoïnae). Specimens were collected in Brittany and were kept for a few days in individual boxes placed in a seawater aquarium to enable them to reload their photogenic charges fully. They were anesthetized with $10^{-4}$ tricaine metasulfonate (MS 222, Sandoz, Basel) and their elytra were isolated by provoking autotomy. Isolated elytra were rinsed several times in seawater, individually checked under a fluorescence microscope for complete absence of green fluorescence from the photogenic area (λ max excitation, 460 nm; λ max emission, 529 nm) and used within a few hours. Such "virgin" elytra are known to possess a full photogenic charge.

Fast-freeze fixation was done with the prototype of an apparatus (Cryobloc) developed by Escaig et al. (14, 15), now constructed by Reichert-Jung (Vienna), and now commercialized by Cambridge Instruments, Inc. (Monsey, NY), in which a polished, pure copper block is cooled to about −260°C

by liquid helium inside a vacuum chamber to avoid condensation. The specimen was slammed onto the surface of the block by an electromagnet within 15 ms of the vacuum chamber venting. Measurements with thin thermocouples have shown that the surface of the specimen freezes in a matter of milliseconds (15).

An elytrum was placed on a moistened disk of filter paper over a thick layer of urethane foam and a layer of mica was interposed between the paper and the foam. Two isolated platinium wires passing through these supports supplied the electric stimulation to the dorsal side of the elytrum. The stimulus was a 5-ms square pulse delivered every second by an SD5 stimulator (Grass Instrument Co., Quincy, MA). Its intensity was progressively increased until the first flash appeared, and then maintained at this threshold value. The experiments were done in a lightproof box which enclosed the top of the freezing apparatus. The flashes emitted upon stimulation were directed via a small mirror and a light guide to a photomultiplier tube (RCA IP21, RCA New Products Div., Lancaster, PA) and displayed on a storage oscilloscope (5103 NDS Tektronix, Inc., Beaverton, OR).

Freeze substitution: after fast freezing, the specimens were stored in liquid nitrogen and transferred for 3 d into dried acetone containing 5% $OsO_4$, at −90°C in the presence of a molecular sieve (No. M-0133, Sigma Chemical Co., St. Louis, MO). The specimens were then gradually thawed, first to −30°C and maintained for 2 h at this temperature, then thawed to room temperature, rinsed with pure acetone, cleared in propylene oxide, and embedded in Epon. Frontal and transverse sections were cut with a diamond knife, contrasted with uranyl acetate and lead citrate, and observed with a Philips EM 300 electron microscope.

Fixation was done either on virgin elytra without stimulation, or on stimulated elytra under repeated stimulation (1 Hz), during the period of facilitation or later, at the end of the phase of decay of the typical emissions.

## Results

The FFFS method failed in about a third of the attempts, probably when the flatness of the elytrum was altered by the stimulating electrodes. This limited the present study to a qualitative comparison of virgin versus stimulated elytra. When it was successful, FFFS provided a morphologic preservation of unchallenged quality in the top 15 μm, which were devoid of ice crystals and included the epithelial cell bodies. Here the details were extremely sharp; there were no "empty" cytoplasmic areas, no indication of swelling, no large vesicles, and no myelinlike bodies such as those usually observed around photosomes after conventional glutaraldehyde and osmium fixation. All membranes were sharply trilamellar. Deeper in the preparation, crystallization artifacts appeared as meshes of increasing size and as membrane disruptions, but very progressively, so that they were easily recognized. The general features of the photocytes and of their characteristic photosomes have already been extensively described and were also easily recognized (1, 8, 9, 24,

*Figures 1 and 2.* (Fig. 1) Virgin elytrum. A general view in a transversal section of the photogenic epithelium on the lower side of the elytrum. The single-layer photogenic epithelium is characterized by large paracrystals of ER (*PER*), i.e., the photosomes, which are the microsources of the luminescence. They are surrounded by arrays of intermediate ER (*IER*). Four photosomes are enclosed in pouches which protrude into the wide extracellular space (*E*) of the middle compartment. Another photosome is located at the level of the nucleus (*N*). They respectively belong to the outer and inner rings of photosomes observed in vivo. A few tentacle-like pseudopods (*T*) are in contact with the pouches. An intercrossing system of pillars (*P*) running through the middle compartment connects the epithelial cells of the opposite side of the elytrum. Inside the cell bodies, plasma membrane invaginations form numerous extracellular spaces which almost reach the cuticle (*C*). At the top of the figure, the progressive reticulation of the extracellular space is the first sign of a crystallization artifact. (*G*) Golgi area. Bar, 2 μm. (Fig. 2) Stimulated elytrum, fixation during the period of facilitation, after the first 10 flashes. Frontal section at the level of the inner ring of photosomes. These photosomes are the last to be coupled during facilitating responses. Pillars (*P*) coming from the cells of the opposite side are anchored by desmosomes along the cell membrane invaginations that form extracellular spaces (*E*). A photosome not yet coupled (*PER 1*) is connected to the nuclear envelope. Another photosome (*PER 2*) is connected by the IER to two relatively short DER saccules (*arrowheads*) established along the plasma membrane of nearby invaginations. Junctional complexes are fully developed around the third photosome (*PER 3*), which is enclosed in a lateral digitation of the photocyte membrane. Along this boundary, digitations and tentacles (*T*) are intertwined. They are filled with IER connected to long dyadic saccules. Between the fibrillar material which fills most of the cytoplasm, strands of ER connect the photosomes to each other and to the nuclear envelope. (*m*) Mitochondria; (*N*) nucleus. Bar, 2 μm.

*Figures 3 and 4.* (Fig. 3) Virgin elytrum. Transverse section. In this specimen, the IER is particularly well developed. It is either connected to the photosome (*PER*) enclosed in a pouch, or consists of free cytoplasmic strands, particularly in a protruding pseudopod. Nevertheless, this IER does not reach the plasma membrane except at the tip of the pseudopod and there are no dyadic saccules. Pillars (*P*) are filled with fibrillar material. The ascending ones arise from the cell bodies in the direction of the opposite side of the elytrum, and the descending ones end in desmosomes (*d*). (*C*) Cuticle; (*E*) extracellular space. Bar, 1 μm. (Fig. 4) Virgin elytrum. A Golgi apparatus (*G*) is apparently involved in the formation of the intermediate ER (*IER*). Short saccules parallel to the plasma membrane are probably dyads in the early stages of formation (*arrowheads*); they bear ribosomes and one is connected to rough ER (*RER*). (*d*) Desmosome; (*m*) mitochondria; (*\**) gap junctions. Bar, 0.5 μm.

32). Nevertheless, FFFS brought out additional morphologic details and therefore allowed better comprehension.

## Photogenic Cells

Like all other epithelial cells of the elytrum, the photocytes lie on a continuous cuticle. The lateral cell surfaces are interdigitated like pieces of a puzzle and linked by desmosomes, septate junctions, and numerous gap junctions (8). The upper cell surfaces have no basal lamina and bathe directly in the wide extracellular space of the middle compartment of the elytrum. The topography of these upper surfaces is complex (Figs. 1 and 2). It shows several types of extensions which can be classified as pillars, pouches, and pseudopods or tentacles. Between these extensions, funnel-shaped invaginations penetrate deep into the cell bodies.

The pillars cross the entire middle compartment and are anchored by desmosomes in invaginations of the epithelial cells on the opposite side of the elytrum. Conversely, pillars originating from these cells on the opposite side are similarly fastened by curved desmosomes all along the invaginations of the photocytes. As a mechanical framework, filled exclusively with fibrillar material, this intercrossing system accounts for most of the thickness of the elytrum.

Observation in toto by fluorescence microscopy (3, 4, 6) had shown that the photosomes are basically disposed along two concentric rings within each photocyte, the inner ring being located around the nucleus, and the outer one, slightly above, forming the hexagonal pattern described previously (4). Here, single or clustered photosomes of this outer ring were seen to be enclosed in pouches which protruded in

varying degrees into the middle compartment. Some were connected to the cell body by a slender neck. Pouches of adjacent cells intermingled and formed a line along the edges of the photocytes. Adjacent pouches exhibited long gap junctions.

Among the invaginations, pillars, and pouches, the surface of the photocytes was extremely tortuous and apparently flexible and deformable. Here, FFFS preserved a whole series of pseudopods. Some were very slender and wrapped around the pouches or joined together as if they had been caught frozen in the middle of a whirling fluid movement. We called the longest pseudopods tentacles and they will be described in greater detail when we consider the stimulated cells in which they are particularly developed.

The fundamental cytoplasmic components of the photocytes are the same as in nonphotogenic epithelial cells. They comprise relatively few scattered mitochondria, short irregular profiles of smooth and rough ER, several Golgi apparatuses, often numerous lysosomes and dense bodies, patches of glycogen, and a few microtubules. Numerous fibrillar bundles fill up the pillars, occupy large portions of the cytoplasm in the cell bodies, and are connected to the desmosomes or the cuticle. The only difference between nonphotogenic and photogenic cells is the presence of photosomes in the latter. Photosomes are never far from a plasma membrane, be it that of a pouch, an invagination, or a digitation of the lateral surface.

After FFFS, the photosomes were far more electron dense than after usual chemical fixation. This was due to improved preservation of the product held in the curvature of the tubules, while their lumen appeared empty. The photosomes had the same appearance in virgin and excited cells and there was no sign of consumption of the photogenic reserves. Mitochondria also had an unusually dense matrix. Lastly, FFFS preserved a network of smooth ER, the IER, which had not been noticed earlier after conventional chemical fixation. This IER arose directly from the constitutive tubules of the photosomes but no longer had their characteristically regular diameter and curvature. The change was sudden at the edges of the photosomes, as if the paracrystalline organization were metastable (Fig. 3). The striking characteristic of the stimulated elytra was that the IER connected numerous long terminal saccules of DER. In virgin elytra, on the contrary, DER saccules were fewer, shorter, and often not connected with the IER. For example, 55 DER saccules were observed along the plasma membrane of the pouches which enclosed 41 randomly selected photosomes in virgin elytra, and their mean length was $1.08 \pm 0.66$ μm. In stimulated elytra, there were 122 DER saccules around the same number of photosomes and the mean of their very diverse lengths was $1.54 \pm 1.26$ μm. The overall length of the DER relative to the perimeter of the photosomes better expressed this difference. It was 2.3 times greater in stimulated elytra.

### Virgin Elytra

The IER varied considerably depending on the specimens. In some cases, it only formed a limited halo around the photosomes, or was even restricted to one or two of their facets; it ended abruptly in the cytoplasm and hardly ever reached the plasma membrane. In other specimens, the IER strands were more developed; they partially filled digitations on the lateral surfaces of the cells, as well as the pouches, and al-

most reached the plasma membrane (Figs. 1 and 3). Golgi apparatuses were apparently involved in this IER development (Fig. 4), and it seemed that vesicles arising from the edges of the Golgi saccules joined the growing strands.

The DER varied greatly in the pouches that enclosed the photosomes. ~10% of the pouches displayed long DER saccules (2–4 μm) with an upper side parallel to the plasma membrane while the other was connected to the IER. The pattern was sometimes repeated symmetrically in an adjacent pouch or tentacle and constituted a triad. These features might characterize the precoupled photosomes. In contrast, the DER was completely absent in ~22% of the pouches, in which the IER ended without any further differentiation (Fig. 3). DER saccules were present in the other pouches. However, they were relatively short (0.2–1.6 μm) and they never exhibited the symmetrical arrangement of triads. About a fourth of them had no connection at all with the IER, which was sometimes far away, and bore ribosomes on their lower side. The smallest isolated DER saccules were found near the Golgi apparatuses and were connected to short cisternae of rough ER (Fig. 4).

Lastly, we must mention the case of a few elytra in which the IER was not only extensive but also connected to large and numerous DER saccules, thus creating a situation similar to that of stimulated elytra (not shown). This was probably due to spontaneous luminous activity during the manipulation of these elytra prior to fast freeze fixation.

### Stimulated Elytra

Stimulated photocytes constantly exhibited considerable development of the IER and the DER connected to it. In all the pouches, the entire cytoplasm between the photosomes and the plasma membrane was now filled with IER. The IER cisternae formed a continuous fairly irregular network oriented radially in the direction of the nearest cell membrane border. There were always several perpendicularly oriented DER saccules connected to the IER and running parallel to the plasma membrane, and their length was often >5 μm (Fig. 5–8). There were no longer any isolated DER saccules bearing ribosomes.

The width of the intracisternal space (50 to 70 nm) was constant all along the DER saccules, and the space between the outer DER surface and the cell membrane was also constant (50–70 nm) (Figs 10–12). It was electron lucent, and no foot process or bridge of any kind was observed to cross it. Dyadic saccules followed the bends and curvatures of the plasma membrane.

DER was almost exclusively present along those portions of the plasma membrane which, on the outer side, bordered on narrow extracellular spaces. In this situation, the junctional complexes were mostly symmetrical, forming triads or even more complicated figures (Figs 9–12). It appeared that the narrow extracellular spaces could be created by a variety of situations. One was provided by the deep funnel-shaped invaginations which penetrated the photocytes; the junctional DER complexes which developed along their sides were connected by IER to photosomes of the inner ring (Fig. 2). A second such situation occurred along the lateral cell boundaries (Fig. 2) as well as between the pouches arising from adjacent cells (Fig. 9). All of them were closely apposed and locally connected by gap junctions (Fig. 10). A third form of narrow extracellular space formation, which
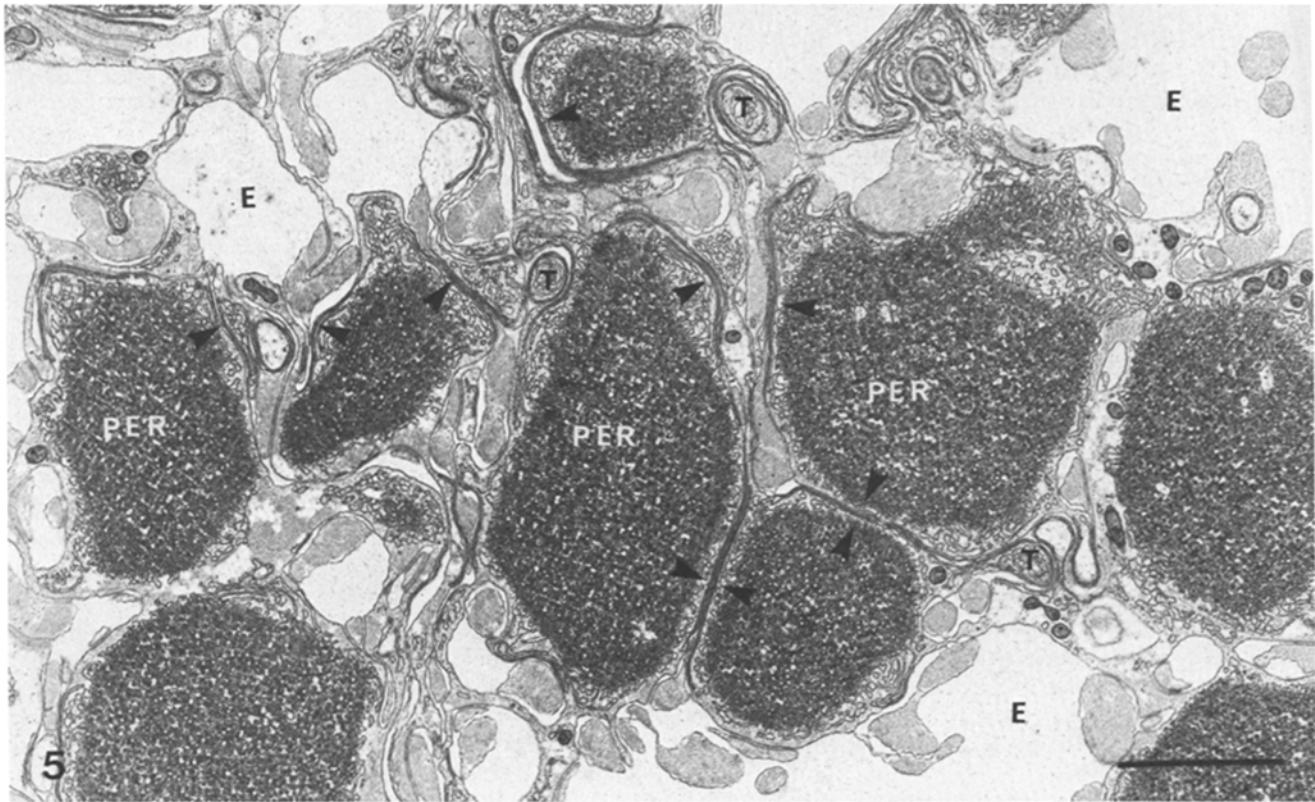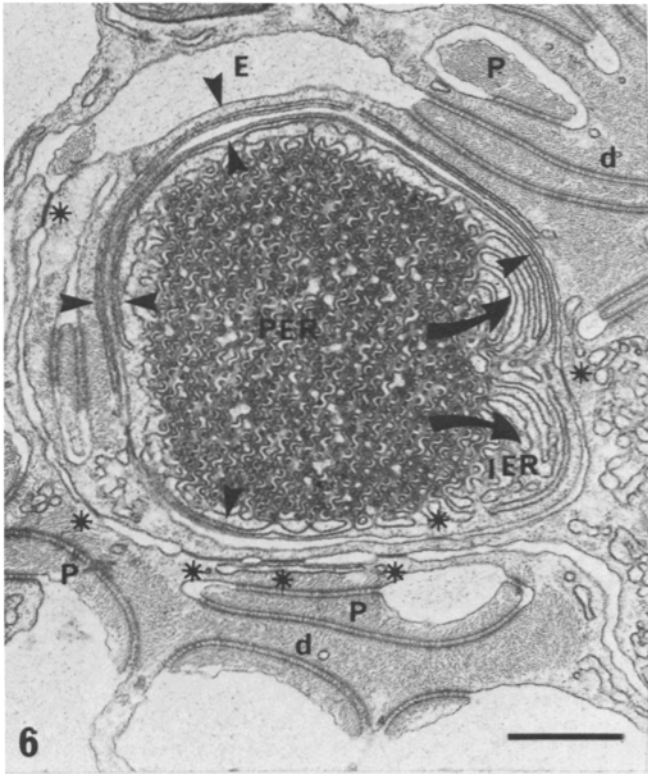
*Figure 5.* Stimulated elytrum. Fixation at the end of a typical emission series. Frontal section at the level of the outer ring of photosomes. Photosomes (*PER*) are individually enclosed in pouches and they are all connected to numerous long terminal dyadic saccules (*arrowheads*) parallel to the plasma membrane. Adjacent pouches and tentacles (*T*) are intermingled. (*E*) Extracellular space. Bar, 2 μm.

obviously implied dynamic processes, was created by the pseudopods and tentacles. The latter arose between or from the pouches, and grew freely in the extracellular space. It seemed that growing strands of IER were initially responsible for the protrusion of pseudopods as if they were pushing out a fluid plasma membrane (Figs. 6 and 7). Thereafter, the pseudopods were apparently liable to considerable elongation; they became slender tentacles terminated by a swollen, oval head. During this elongation, they often became so flat that they contained only one strand of ER (Fig. 8). Unlike the pouches, which are probably constant structures, pseudopods and tentacles never contained bundles of fibrillar material or mitochondria. DER and IER, or sometimes patches of glycogen, were always present in their enlarged tips. A continuous network of IER could be followed in several instances in the neck of the tentacles up to its connection with a photosome. However, this IER network often appeared to be interrupted probably simply because the section missed parts of its track. Some tentacles wrapped themselves around

a pouch (Figs. 6 and 9). Others joined together and twisted into a coil, so that their enlarged heads, apposed side by side, formed Yin-Yang figures (Figs. 8 and 13). Occasionally, more than two tentacles were involved in the same coiling process, which resulted in an intricate interdigitation. Gap junctions were also observed between adjacent tentacles (Fig. 6). All these pairing processes resulted in the creation of narrow extracellular spaces.

To sum up, FFFS revealed significant differences between virgin elytra, in which most photosomes were considered to be in the uncoupled state, and stimulated elytra. Only the latter displayed impressive development of the DER and connected IER. A striking feature of these junctional complexes was that they mostly occurred symmetrically in triads, at the level of the narrow extracellular spaces or even along gap junctions. The narrow spaces were either built-in, like the invaginations, or resulted from the combination of pseudopods and tentacles arising from adjacent cells and pairing in a transient association.

*Figures 6–8.* Stimulated elytrum. Fixation at the end of a typical emission series. Representative aspects of photosome coupling. (Fig. 6 and 7) Photosomes (*PER*) enclosed in pouches around which whirling tentacles (*T*) create a complex labyrinth of narrow extracellular spaces (*E*). Intermingled tentacles from adjacent cells are linked by gap junctions (*). Intermediate ER (*IER*) extends from the well-organized tubules of the photosomes and connects the long terminal dyadic saccules (*arrowheads*). The IER seems to grow actively in some places where several saccules are piled up (*curved arrows*). (Fig. 8) The enlarged head of the tentacles filled with IER or exceptionally, with glycogen (*gl*), and bordered by DER saccules. Most of the DER are symmetrically arranged in triads in the pouches and adjacent tentacles, or between adjacent tentacles. The continuity of the IER network is intermittent in the neck of the tentacles. (*P*) Pillars; (*d*) desmosomes. Bar, 0.5 μm.

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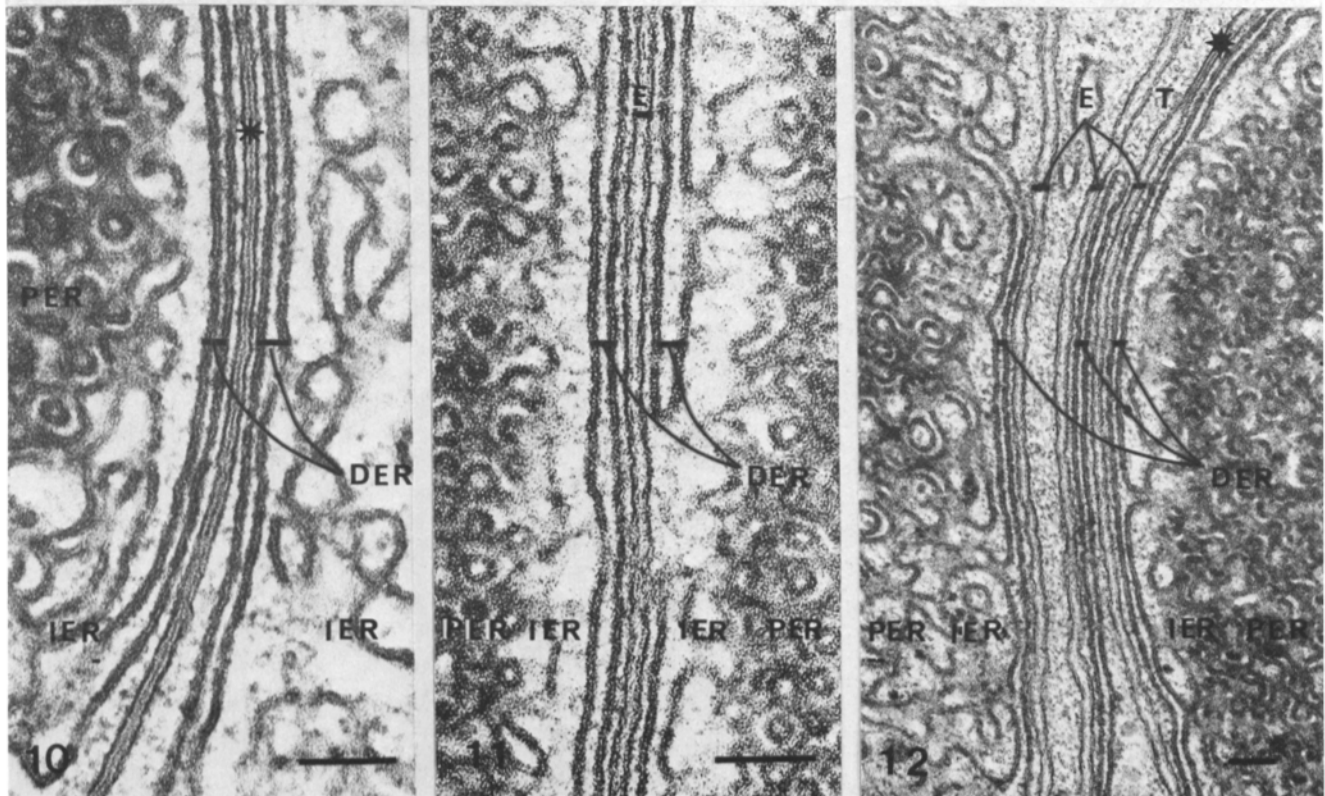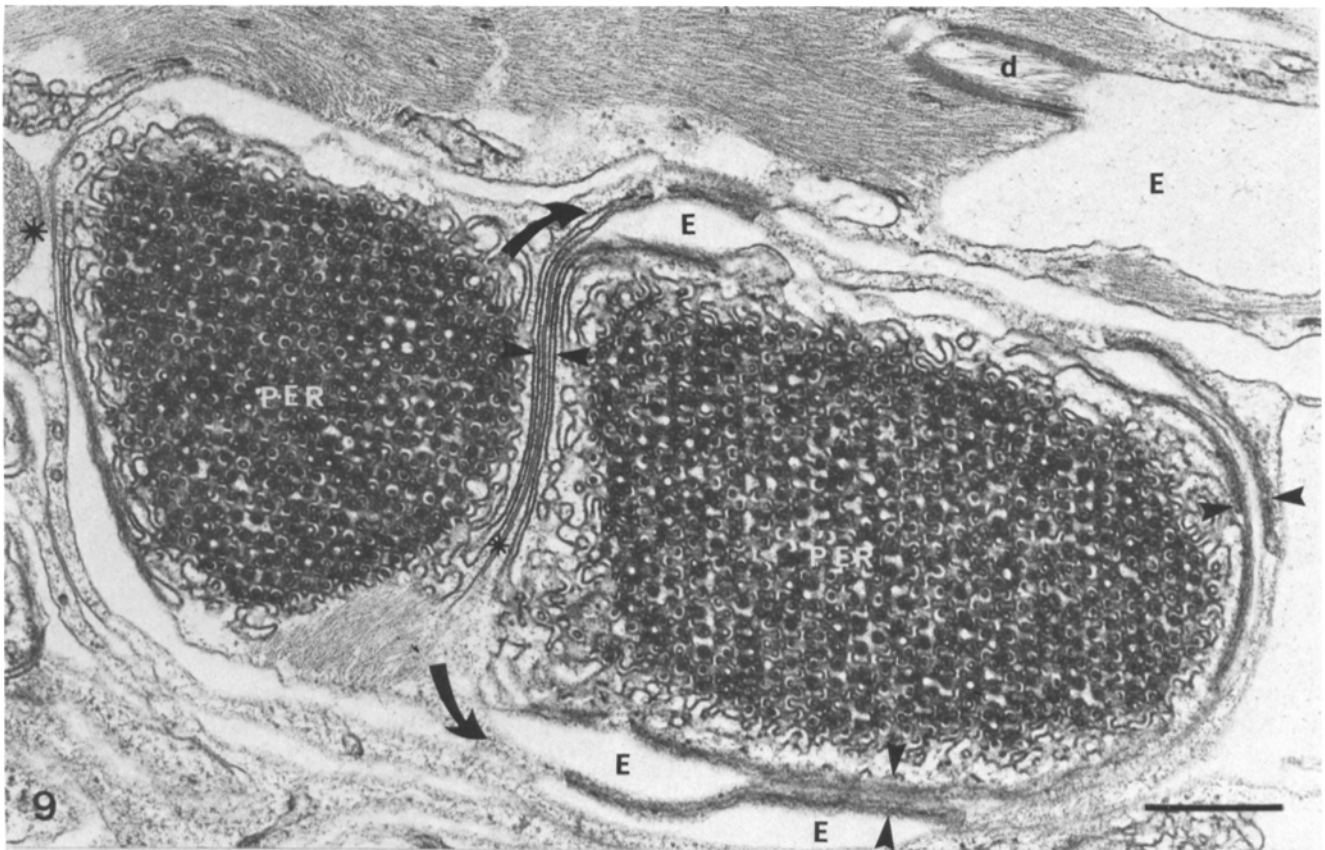
*Figures 9–12.* Stimulated elytra. Fixation at the end of a typical emission series. Junctional complexes between adjacent pouches. (Fig. 9) Two pouches which each contain a photosome (*PER*) and are connected by a long gap junction (*). In addition, a slender pseudopod arises from the left pouch (*curved arrows*) and embraces the right one. Long dyadic saccules (*arrowheads*) extend symmetrically and form a triad. (Fig. 10) Detail of the triad along the gap junction. Short strands of intermediate ER (*IER*) connect the paracrystalline ER of the photosomes (*PER*) to terminal saccules of dyadic ER (*DER*). Unit membranes are clearly defined. (Fig. 11) Similar triad along a narrow
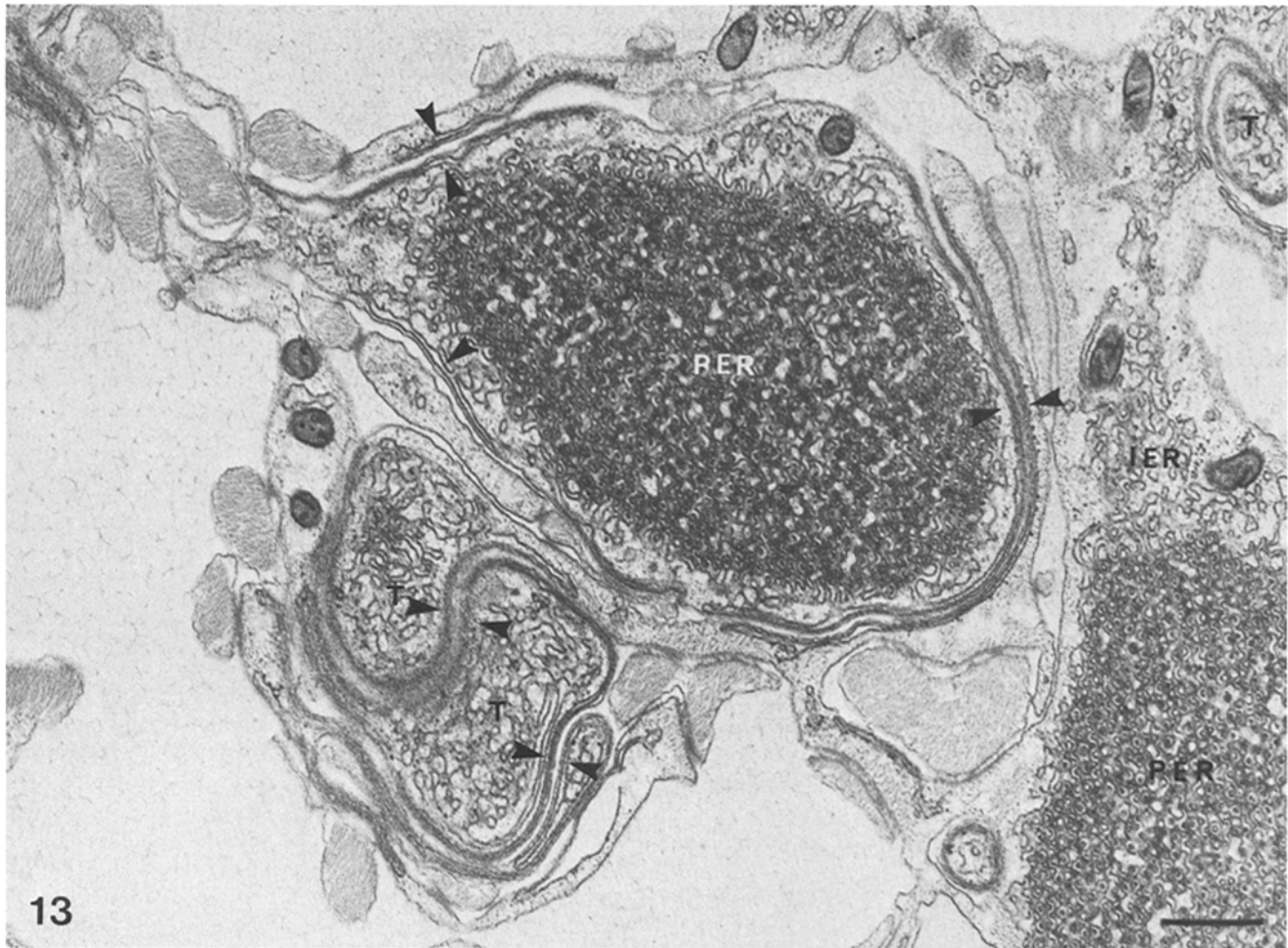
*Figure 13.* Stimulated elytrum. Fixation at the end of a typical emission series. In the wide extracellular space of the middle compartment, the enlarged heads of tentacles (*T*) join and twist together in Yin-Yang figures, forming narrow extracellular spaces. Triads (*arrowheads*) extend at the level of these narrow spaces. Note the continuous strand of IER which joins the photosome (*PER*) on the right of the figure. Bar, 0.5 μm.

## Discussion

In the problematics of the bioluminescent system of the scale worm, the word "coupling" has two meanings which must be clearly distinguished. First, when the word applies to the coupling of excitation and bioluminescence, it refers to the path along which the information travels, eventually triggering the flash of a photosome. The second meaning of coupling is the switch-in action of an intracellular portion of the same path by which the excitation–bioluminescence coupling is completed at the level of the individual photosomes. The existence of such a transient mechanism has been postulated to explain the correlation established in vivo between recruitment and facilitation (4). The present study strongly suggests that (*a*) the morphologic support of the coupled state is indeed a dyadic junctional complex which is adjacent to the plasma membrane on the one hand and linked to the photosomes on the other, and that (*b*) the differentiation of this

junctional complex, which is completed in a surprisingly short time, involves a dynamic process of membrane transformation on a surprisingly large scale. Before discussing the functional and dynamic aspects of coupling, the reliability of the present observations has first to be considered.

### FFFS

Because fast-freeze fixation is completed in milliseconds, it is the only method capable of immobilizing fast transient cellular processes. Its most interesting applications, particularly by Heuser et al. (20–22), have shown the capturing of successive images of a precisely timed cytophysiological event such as synaptic transmission. The bioluminescent elytral system, with its fast luminous responses, stepwise recruitment, and single layer of photogenic cells, provides material well adapted to this technique. The epithelial cells are small enough to fall into the 15-μm range which is free

extracellular space (*E*) between adjacent pouches. (Fig. 12) A more complex arrangement with an interposed tentacle (*T*) bearing a short gap junction (*). Bars, 0.5 μm (Fig. 9) and 0.1 μm (Figs. 10–12).

of ice crystals and in which preservation can be exceptionally good (23, 29). This quality is first of all due to the rapidity of the fixation, which avoids all the transformations and deformations that can occur during the slow penetration of a fixative solution between cell death and chemical fixation (18). Secondly, the FFFS technique eliminates the artifacts resulting from osmotic pressure, because the dehydration is done while the water content of the preparation is in a solid state. As far as the IER is concerned, its preservation was so poor after the usual glutaraldehyde and osmium fixation that it remained undetected until FFFS gave clear evidence of its characteristic appearance and location. It is therefore plausible to consider that the ionic concentration inside the IER cisternae might differ markedly from the concentration in the surrounding cytoplasm or in other compartments of the ER, and that this might explain the transformation of the IER into either PER or vesicles and myelinlike bodies when it is penetrated by a liquid fixative adjusted to an average osmotic pressure.

Although the present study firmly established the difference between virgin and stimulated elytra, the latter presenting at least twice more junctional ER, it did not allow step-by-step analysis of the differentiation of the coupling system. One reason was due to failures in the reproducibility of our FFFS method which spoiled certain series of experiments on successive elytra of the same animal. Another reason was the unexpected variability encountered in virgin elytra from different individuals. This variability might reflect different stages of replenishment after possible previous luminescent activity. Conditions for the quantitation of the membrane surfaces forming dyad junctions and of tentacle differentiation would be more favorable in a species like *Acholoe*, in which all the steps can be studied in the same elytrum because of the progressivity of the territorial extension during facilitation.

### Excitation–Bioluminescence Coupling and the Conducting ER

The ER of the photogenic cells is exceptionally abundant. It forms a continuous internal compartment which exhibits, in a fairly linear order, a series of morphologic differentiations that fall into two functional groups.

What is conventionally defined as the ER includes the outer membrane of the nuclear envelope, smooth and ribosome-bearing saccules, and associated Golgi apparatuses (G) and lysosomes (L), which together form the GERL, as described by Novikoff et al. (27, 28). This GERL is involved in secretory processes, including those of the photosomes and of the cuticle, and generally localized in the perinuclear area of the photocytes. It is particularly abundant in growing and regenerating elytra, in both of which direct continuity between the tubules of the photosomes and the conventional ER is frequent (24). Although little is known about the elaboration and turnover of the photogenic products stored in the photosomes, these are probably relatively slow processes, occurring on a time scale of minutes or hours.

The three other parts of the ER, namely the PER, IER, and DER, are undoubtedly involved in the fast event of the flash, which occurs in response to stimulation after a delay as short as 1 ms. In that the present study tends to prove that the firing photosomes are exclusively those coupled with the plasma membrane by a junctional complex comprising the

IER and DER, this complex must indeed constitute the internal route by which the excitation–bioluminescence coupling is completed.

As already proposed (31, 32), the topographic characteristics of the elytral junctional ER are comparable to those of the striated muscle, in which the sarcoplasmic reticulum lying alongside narrow invaginations of the plasma membrane (T system) forms dyads or triads. They also resemble the characteristics of the peripheral junctional sarcoplasmic reticulum of the heart muscle, apposed to the surface sarcolemma (17). In a prochordate muscle described by Cavey (11, 12), the comparison is further completed by the presence of tubular paracrystals of sarcoplasmic reticulum, called polylamellar aggregates, which look exactly like photosomes. Thus the muscle and the bioluminescent elytral systems can be considered as homologous, at least as far as the ER or sarcoplasmic reticulum are concerned. In both cases, similar junctional complexes form a continuous membrane compartment with the same successive differentiations, and both are involved in a fast response consisting of either contraction or bioluminescence. It remains to be established whether these systems involve similar mechanisms.

Compared with the muscle system, the junctional complex in the photocytes is, as it were, overdeveloped. The dyads extend parallel to the plasma membrane over a length of several micrometers, the intermediate reticulum is extensive and irregular, and there are several ways in which the cell forms narrow extracelullar spaces similar to the T invaginations. Coupling is not a built-in device, but a dynamic and pleiomorphic process whose functions raise several questions.

The first is the mechanism by which depolarization (or some other change in the surface membrane) crosses the gap between the outer leaflet of the DER and the surface membrane. In the absence of any visible foot processes such as those described in muscle triads (16, 33), the hypothesis of a chemical transmission by a second messenger across the gap (34) will have to be tested. The second question concerns the kind of electric or ionic signal that is transmitted along the IER to the photosomes after the gap has been crossed, and how this signal is able to trigger the production of oxygen radicals, which in turn will trigger the luminous reaction of polynoidin (25). The third question is the polarity of the junctional complexes. Photosomes are the reactive pieces of the system, but not necessarily the terminal ones, and it is possible to consider that the information propagated by the ER passes through the photosomes and then continues further on. As its circuit builds up, the conducting ER provides a continuous pathway which connects more and more photosomes inside the cell. It is even possible that information might pass from cell to cell through the triads, when they extend their symmetrical junctions over the gap junctions of the lateral contacts, adjacent pouches, or entwining tentacles.

This concept explains why the photosomes of the outer ring are ignited first (4), the internal route being much easier to build and to follow at their level than deeper in the cell. It also explains the territorial extension which propagates the coupling from cell to cell (5) as well as inside each cell (4). Furthermore, it explains the stepwise increase, flash after flash, in the delays between the stimulation and the response during a typical emission (2, 10), as a result of the increasingly long pathway of exhausted photosomes along which the stimulation must travel to reach more recently coupled photosomes which are still reactive. Lastly, the conducting ER

circuit would account for the two major differences between the nonphotogenic and the photogenic cells of the same conducting epithelium, namely, the occurrence of photosomes and the character of their action potential (8, 9, 19). A recent electrophysiological reinvestigation of the system (Bassot, manuscript in preparation) showed that the action potential associated with the emission of a flash increases progressively its amplitude during the period of facilitation, period now characterized by a spatial increase of the network of conducting ER.

## Differentiation of the Junctional Complex

In vivo studies have shown that recruitment proceeds along definite spatial pathways at a speed that depends on the stimulation frequency (2–4). The results of FFFS strongly suggest that coupling involves highly dynamic membrane processes occurring at quite a high level of cooperative integration, in that the junctional complexes link the PER, IER, DER, and plasma membrane in a sophisticated architecture.

Elements of this architecture certainly preexist in the resting system. Photosomes are permanent structures which might be formed by Golgi apparatuses (31), by crystallization of arrays of smooth ER (24), or even by newly formed ER emerging from the nuclear envelope, as described in UT-1 cell paracrystals (30). The Golgi systems observed in some virgin elytra appear also to participate in the formation of the IER. However, the DER is apparently formed independently, from short, rough ER saccules arranged along the plasma membrane (Fig. 4). A few complete junctional complexes are even found in virgin elytra. They account for the precoupled photosomes ready to respond to the initial stimulation.

Nevertheless, most of the junctional complexes must be completed or built up very rapidly during the period of facilitation. Active mechanisms involving membrane recognition and transformation must occur for the DER saccules to differentiate and establish the specific cooperation reflected by the even spacing between them and the plasma membrane. It is certainly significant that the DER saccules almost always develop opposite capillary extracellular spaces, whatever the diversity of their constitution. Assuming the existence of a coupling inducing factor, these parts of the plasma membrane would act as specific gates. The diffusion of the coupling inducing factor would then explain why the IER chooses the shortest way of reaching a specific region of the plasma membrane. It would also account for the occasional observation of superimposed piles of DER and IER saccules (Figs. 5 and 7) as an exaggerated effect. Lastly, it would explain reinforcement in terms of an increase in the number and surface of the junctional complexes which serve a given photosome under the influence of the same factor.

Reinforcement and decoupling also require more studies. At present, it seems that the first event responsible for decoupling could be a dislocation interrupting the continuity of the IER. The dedifferentiation of the DER in resting elytra is probably of later occurrence. However, the decoupling mechanism is not the result of a collapse, as proposed by Sommer et al. (35, 36) for the intermediate segment of the sarcoplasmic reticululm.

In conclusion, the conducting ER allows the transport of a triggering information to specific sites inside the cell. The coupling device is transient and is induced to differentiate by the stimulation itself. It not only allows a rapid reaction, triggered from outside, in a specific cell compartment, but also anticipates the next stimulation in preparing an enhanced response. The existence of transient junctional complexes of ER was established in the bioluminescent system of the scale worm thanks to their striking effect on luminescence and to the use of a fast method of fixation. It remains to be seen whether this new modality of stimulus–effector coupling is of more general occurrence.

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