SYNAPTIC ACTIVITY OF FROG RETINAL PHOTORECEPTORS
A Peroxidase Uptake Study

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ABSTRACT
The uptake of horseradish peroxidase (HRP) into membranous structures, detectable by light and electron microscopy, is used here to monitor the synaptic activity of photoreceptors of isolated frog retinas maintained in the dark or under various illumination conditions. The major findings are: (a) Neurotransmission from photoreceptor terminals seems to involve the same types of endocytic membrane-retrieval processes that occur at other nerve terminals. Presumably, the endocytic processes compensate for exocytic events associated with neurotransmission. The retrieved membrane is “recycled” to form vesicles. Some of these accumulate near the synaptic ribbons, perhaps indicating reutilization for exocytosis. On the other hand, some retrieved membrane evidently is degraded via multivesicular bodies that appear to undergo “retrograde” transport from the receptor synapses to the myoid regions. (b) Photoreceptor terminals take up much HRP in the dark. Steady illumination markedly decreases uptake by rods. Uptake by cones is notably reduced only at illumination intensities higher than those that have maximal effects on rods. (c) The decrease in rod HRP uptake with light is reversible when retinas are allowed to adapt to the dark, if the light exposures used were at intensities that bleach very little visual pigment. Such “recovery” is not observed after light exposures that bleach a considerable amount of visual pigment. The cones recover their dark levels of HRP uptake even after light exposures that bleach considerable amounts of visual pigment. The changes in HRP uptake that we observe parallel expectations for photoreceptor synaptic neurotransmission derived from indirect physiological evidence.

Secretion by many cell types occurs through exocytosis, the fusion of the membrane that encloses material destined for release with the plasma membrane at the cell surface. Subsequently, membrane is retrieved from the cell surface by endocytic-like processes (reviewed in references 26 and 27). For neurons, the evidence that such “compensatory” endocytosis is coupled to transmitter release is based chiefly on cytochemical studies of the uptake of extracellular tracers. Stimulating neurotransmission in lobster neuromuscular junctions (25), frog neuromuscular junctions (22, 31), cultured mouse spinal cord neurons (54), and other systems (32, 40, 58) results in a marked increase in the uptake of extracellular tracers, such as horseradish peroxidase (HRP) and dextrans, into vesicles and other membranous structures within the presynaptic terminals. Nonstimulated
preparations show comparatively little tracer uptake.

The observed relationship between transmission and endocytosis suggests that tracer uptake could be utilized as a method for evaluating levels of synaptic activity in nervous tissue difficult to study by more conventional means. In the present investigation, we have employed HRP to monitor the synaptic activity of frog retinal photoreceptors in the dark and under various illumination conditions. We found changes in the level of HRP uptake into vesicles and other membranous structures with illumination which strongly suggest that photoreceptor terminals are active in the dark and that varying levels of light decrease the activity of rod and cone terminals to varying degrees. Our findings also suggest that some of the membrane “retrieved” from the cell surface is reutilized to form seemingly functional synaptic vesicles and that some is degraded in multivesicular bodies that undergo “retrograde” transport to the myoid region.

Preliminary reports of these findings have been published (48-52). Subsequently, an abstract was published confirming the utility of the approach in studies on skate preparations (46).

MATERIALS AND METHODS

Preparation of Isolated Retinas

Frogs (Rana pipiens) about 3 inches in body length were dark adapted for 24 h before each experiment to facilitate isolating the retina from the pigment epithelium. A dark-adapted frog was decapitated and one or both eyes enucleated. The anterior portion of the eye was removed by cutting below (behind) the ora serrata, and the retina was carefully teased away from the pigment epithelium in a dish of Ringer’s solution (111 mM NaCl, 2.3 mM NaHCO3, 2 mM KC1, 1.1 mM CaCl2, and 5 mM glucose (16), pH 7.0). The retina was floated, receptor side down, on a Ringer’s soaked pad. It was next transferred to a plastic chamber which has a moat filled with Ringer’s solution that surrounds the retina and its intensity was adjusted with neutral density filters. The illumination covered the entire retina, and its intensity was adjusted with neutral density filters. The illumination intensities used for the tracer experiments, expressed as the log of the fraction of the full intensity, were: dark; -5.1; -3.3; -2.1, and zero (full intensity i.e., 130 ft-candles).

Tracer Experiments

The retinas were presoaked in the dark in Ringer’s solution containing 0.5% HRP (Sigma Chemical Co., St. Louis, Mo.; Type II) for 3 min before floating on a cotton pad. The retinas were placed in the experimental chamber, and illumination of the appropriate intensity was presented. Experiments lasted from 15 to 120 min. Experiments were terminated by a 3-min rinse in HRP-free Ringer’s in the dark.

Fixation

After the brief rinse, the retinas were fixed by immersion in 2.5% glutaraldehyde (47) in 0.1 M cacodylate buffer (pH 7.2) with 3% sucrose and 0.25% CaCl2 (34), at room temperature for 30 min and on ice for an additional 90 min. They were then rinsed overnight in cold 0.1 M cacodylate (pH 7.2) with 3% sucrose.

Cytochemistry

Fixed retinas were sliced into small fragments with a razor blade in cold 0.1 M cacodylate in 3% sucrose. The sections were frozen on the head of a freezing microtome for 30-60 s. soaked in the diaminobenzidine (DAB) medium of Graham and Karnovsky (20) without substrate (H2O2) for 30 min and incubated in the complete medium for an additional 30 min at room temperature. Preparations incubated in peroxide-free medium served as controls; these showed no reaction product. Retinas not exposed to extracellular HRP but incubated in the complete medium also showed no reaction product. Enzyme incubations were terminated by rinsing briefly with cold 7.5% sucrose.

Preparation for Light and Electron Microscopy

Tissue was postfixed in cold 1% OsO4 in 0.1 M phosphate or cacodylate buffer (pH 7.2) for 90 min, stained en bloc with uranyl acetate (15), dehydrated in a graded series of ethanols, and embedded in Epon (38). 1-2 μm-thick sections were cut on a Porter-Blum microtome (DuPont Instruments, Sorvall Operations, New- town, Conn.) and examined unstained in a Zeiss phase microscope. Silver thin sections were then cut from blocks, whose thick sections showed uniform reaction product throughout the areas of interest, and examined in an RCA EMU-3F or a Philips 201 electron micro-

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scope. The sections were examined unstained or stained with lead citrate (59). Photographs were taken at magnifications of 7,000-45,000.

**Identifying Rod and Cone Terminals and Myoid Regions**

The criteria we used for identifying the various frog rod ("red" rod) and cone (single and double cones) receptor regions are based on earlier studies (8, 43). In 1-2 μm-thick sections prepared for light microscopy, rod terminals were identified by the presence of long, thin connecting fibers or axons between the terminals and the nuclear regions, which are located in the row of the outer nuclear layer closest to the receptor outer segments. Due to the tortuous nature of the axon, it was often necessary to carefully focus up and down to follow it. Obviously, for many cells only portions of the axon were observed in a given section. Cone terminals were identified by the proximity of the terminals to cone nuclei, which are located in the row of the outer nuclear layer farthest from the receptor outer segments.

In thin sections prepared for electron microscopy, rod terminals were identified by their axons (usually only a portion of the axon was seen in a given section). Cone terminals were identified by their proximity to the corresponding nuclei. Rod terminals are also relatively more electron dense in overall appearance than are cone terminals, and the cones often show a more "patchy" distribution of synaptic vesicles.

Obviously, not every terminal in a given thick or thin section can be unambiguously identified as a rod and cone. We estimate that 50-60% can be so identified. For our tracer uptake analysis, we used only clearly identifiable terminals, and worked with those that were sectioned so as to include a large expanse of the terminal.

The myoid region is the cytoplasmic zone of the inner segment located between the ellipsoid and the nuclear region. Rod myoids were identified by the characteristic position of the rod nuclei and the characteristic shape of their outer segments. Cone myoids were identified by the position of their nuclei and the conelike shape of their outer segments. The presence of an oil droplet in certain cone types also aided in identifying cone myoids.

**Analysis of HRP Uptake**

All experiments were analyzed "blind." At some point during the procedure (usually just before embedding), each retina in a given experiment was relabeled so that the microscopist was unaware of its experimental history. Terminals were identified by the criteria just outlined, and for each terminal the proportion of the synaptic vesicle population labeled with HRP was determined. This was done initially from micrographs by counting vesicles in randomly selected regions within the terminals or by counting the entire vesicle population within each terminal. With experience, we found that we could accurately determine the proportion directly under the electron microscope by surveying a given terminal, forming an impression of the extent of its labeling by inspection, and periodically checking this impression by counting a limited number of vesicles. This permitted us to survey rapidly large numbers of terminals. Fig. 1 presents data comparing estimates of vesicle labeling obtained under the microscope in this way, with numbers derived by systematic counting of labeled vesicles in micrographs of the same terminals. The close agreement between the two sets of numbers indicates that our estimates were accurate throughout the range of labeling proportions important for our study. (There is a large psychophysical literature that predicts that one should be able to make rapid reliable estimates of these types.)

**RESULTS**

**A. Technical Matters**

1. RINSE IN HRP-FREE RINGER’S BEFORE FIXATION IMPROVES HRP LOCALIZATION: As our laboratory previously reported for neurons, the cytochemical demonstration of HRP is markedly affected by the amount of extracellular tracer present at the time of fixation (54). Thus, when retinas are placed immediately into fixative after exposure to HRP, examination of 1-2 μm-thick sections in the light microscope reveals that reaction product is limited to the periphery of the tissue fragments. The 3-min rinse in HRP-free Ringer’s that we use routinely before fixation results in a substantially more uniform localization of HRP reaction product.

2. PHYSIOLOGY AND MORPHOLOGY OF ISOLATED RETINAS WITH OR WITHOUT HRP: The presence of HRP does not appear to affect the physiological status of isolated retinas as monitored by standard extracellular recording methods (29). Threshold measurements of aspartate-isolated (9, 25, 53; 50 mM Na-Aspartate substituted for 50 mM NaCl in Ringer’s solution), mass receptor potentials and electroretinogram (ERG) b-waves were relatively stable for preparations exposed to HRP for up to 120 min in the dark or light. The waveforms and thresholds for dark- and light-adapted receptor potentials and ERG b-waves were also examined in the same retinas before and during HRP-exposure. No significant changes were observed. (While our microscope studies of HRP uptake in aspartate-Ringer’s material were not so extensive as those with normal Ringer’s, we did find the same patterns of change with illumination, and, under given illumination conditions the levels of vesicle labeling did not show statistically significant differences with the two media.)
Figure 1 Estimates of the values for the percent of vesicles labeled with HRP, obtained by our customary examination of the terminals under the microscope, plotted against values obtained for the same terminals by counts made on micrographs. The sample represents rod and cone terminals selected at random from various preparations maintained in the dark for 15, 30, 60, or 120 min.

Isolated retinas incubated in Ringer's without HRP under varying conditions of illumination closely resembled those incubated with HRP in appearance, frequency of structures such as multivesicular bodies (MVB's; see below) and other features examined. With prolonged incubations (2 h in either dark or light with or without HRP), terminals often showed modest numbers of membrane-delimited vacuole-like structures. This may represent deterioration of the terminals (cf. the physiological observations on the effects of anoxia or other "problems" reflected in alterations in the behavior of horizontal and bipolar cells [42]). Vacuolization was especially evident with aspartate-Ringer's.

Table I compares the "density" (number/unit area) of vesicle populations in rod and cone synapses under varying conditions. Changes in density could affect our estimates of the percent of vesicles labeled with HRP, but none were noted.

### Table I

| Min in HRP | Rod | Cone |
|-----------|-----|------|
| Vesicles/μm² | Vesicles/μm² |
| No HRP | 0 | 215 (2) | 252 (2) |
| Dark | 30–45 | 232 (4) | 268 (4) |
| Light (5.1) | 30–45 | 229 (4) | 296 (4) |
| Light (3.3) | 30–45 | 256 (4) | 289 (4) |
| Light (2.1) | 30–45 | 249 (4) | 260 (4) |
| Light (0.0) | 30–45 | 259 (4) | 275 (4) |
| Dark | 15 | 244 (4) | 288 (4) |
| Dark | 30 | 237 (4) | 295 (4) |
| Dark | 60 | 245 (4) | 305 (4) |
| Dark | 120 | 218 (4) | 274 (4) |

The average vesicle density (vesicles per square μm²) in rod and cone terminals is compared for various experimental conditions. The number in parentheses is the sample size (no. of synapses). The vesicle density was calculated from micrographs by randomly selecting at least two regions of unit area (1 μm²) per terminal and counting the vesicles.

B. Peroxidase Uptake

The uptake of HRP into the terminals under the various conditions studied was readily detectable by both light microscopy (Fig. 3) and electron microscopy (Figs. 4–6).
uptake similar to those of rods at the several time points studied.

Uptake into rod and cone synaptic vesicles decreases with illumination. For the rods, this decrease can be observed at relatively moderate intensities (Figs. 3, 4, and 7). Light intensities such as $-3.3$, which are still low enough to bleach very little visual pigment (30), reduce uptake into rods to a low level (cf. Figs. 4 and 5). At lower intensities ($-5.1$), uptake is reduced to intermediate levels (Fig. 7).

The cones continue to show uptake at levels comparable to those of dark preparations at light intensities of $-5.1$ and $-3.3$ (Figs. 3, 4, 6, and 7). Even at the higher light intensities ($-2.1$ and zero) with 30–45-min exposures to HRP, uptake into cones showed little change compared to that of dark preparations (Fig. 7). However, in four separate experiments using 60-min exposures to full intensity light, HRP uptake into cone synaptic vesicles was reduced to 50% of the level observed in 60-min dark exposures (Fig. 8).

2. Recovery: The decrease in the rate of HRP uptake into rod synaptic vesicles with light of $-3.3$ intensity is reversible if the retinas are placed in the dark after the light exposure (Tables II and III). However, after either brief (5 min; Table III) or long (60 min; Table IV) exposure to full intensity light which bleaches about 90% of the pigment (30), rods do not recover their dark levels of HRP uptake even if kept in the dark for up to 60 min. The cones recover their dark levels of HRP uptake even after an initial 60-min exposure to full intensity light (Tables III and IV).

3. Choline: Choline is known to mimic light in that it hyperpolarizes the receptors (7). Retinas maintained in the dark for 60 min in

![Figure 2](image-url)  
**Figure 2** The frequency distribution of HRP uptake into rod terminals maintained in the dark for 15, 30, 60, and 120 min. Each bar represents the category of terminals with the indicated percentage of labeled vesicles. The bar heights were obtained by averaging the frequency of each category (percent of the total population of rod terminals) observed in four separate repeat experiments. $n$ per time point in each experiment was 20–30 terminals.

![Figure 3](image-url)  
**Figure 3** Phase-contrast photomicrograph of a 1–2 $\mu$m-thick section from a retina exposed to HRP for 45 min at a light intensity of $-3.3$. A cone terminal (outlined with straight lines) contains reaction product. The section also includes the nucleus (CN), myoid region (M), and characteristic cone-shaped outer segment (OS) of this cell. Two rod terminals (outlined with arrowheads) contain little reaction product. The rod terminals are attached to the corresponding nuclear regions (RN) by thin axons (arrows). M indicates an HRP-labeled body (probably an MVB; see Fig. 15) in a rod terminal. The bar represents 10 $\mu$m; $\times$ 2,100.

![Figure 4](image-url)  
**Figure 4** Rod (RS) and cone (CS) terminals from a retina exposed to HRP for 45 min at $-3.3$ light intensity. The rod terminal contains few labeled vesicles; the cone terminal shows numerous HRP-labeled vesicles. N indicates a cone nucleus; R, synaptic ribbons; and M, an MVB (lacking HRP). The arrows point to a portion of the rod axon. The bar represents 1 $\mu$m; $\times$ 21,000.
FIGURES 5 and 6 Rod (Fig. 5) and cone (Fig. 6) terminals from a retina exposed to HRP for 45 min in the dark. Both terminals show numerous HRP-labeled vesicles. A few short tubules or sacs are also labeled (T). N indicates the cone nucleus; R, synaptic ribbons; and M, an MVB labeled with HRP. The bars represent 1 μm; x 22,000 (Fig. 5); x 28,000 (Fig. 6).

choline-Ringer's (NaCl is replaced by choline-chloride) show a lower rate of HRP uptake (about 50%) than the dark controls (Table V).

4. HRP LABELING OF RIBBON-ASSOCIATED VESICLES: In both rods and cones, vesicles associated with the synaptic ribbons show HRP labeling (Figs. 9-12). In general, the percentage of the ribbon-associated vesicles found to contain reaction product under varying conditions and at different times was similar to the overall proportion of labeled vesicles in the terminals.

5. HRP LABELING OF MVBS: In typical thin sections, 10-35% of the photoreceptor terminals identifiable as belonging to rods or cones and sectioned so as to include a large expanse of the terminal contained a recognizable MVB (only rarely was more than one present). Extrapolating to three dimensions, this suggests that a large proportion of the terminals contain at least one such body at a given time. In the myoid regions, the cells typically each contained several (2-5) MVB's per thin section cut so as to include most of the region. The overall frequency of MVB's in the terminals and myoids did not change notably under the varying illumination conditions studied, and was similar whether or not the preparations had been exposed to HRP. (Given the variable frequency of MVB's in different sections of a given preparation such statements indicate merely that no very massive changes occur.) There were, however, differences in the proportion of the MVB's that were labeled under varying circumstances. For preparations maintained in the dark, the labeling of MVB's increased with time until at 60 min virtually all the MVB's seen in the terminals (Fig. 14) were labeled and at 120 min nearly all the MVB's in the myoids (Fig. 15) were labeled. The frequency of labeled MVB's in the axons (connecting fibers) of rods (Fig. 13) also increased until at 60-120 min all of these bodies showed reaction product.

Fig. 16 presents data on the frequency of labeled MVB's per thin section of photoreceptor terminals ("synapses") and myoids under varying conditions.
Figure 7 The proportion of vesicles labeled with HRP in rod and cone terminals plotted against the log of the illumination intensity. Retinas were maintained in HRP for 30-45 min. Each data point is the average of the means from four separate repeat experiments (the "error bars" indicate standard deviations). In two of the experiments, aspartate-Ringer's was used in place of ordinary Ringer's for correlation with our physiological experiments (see Results, Section A2); these preparations showed slightly higher average levels of HRP uptake than did their normal-Ringer's counterparts, but the differences were not statistically significant, and the patterns of change with illumination were not affected. a per illumination condition per experiment was 20-30 terminals.

conditions of illumination. No notable changes were found for the cones. With the rods, we consistently found decreases in the frequencies of labeled MVB's and relatively higher frequencies of unlabeled MVB's (cf. Fig. 4) in the myoids and terminals of light-exposed preparations (and in the experiments evaluating postbleach recovery). But, especially for the terminals, the variability was such that the data are only suggestive.

As in many other tissues, structures indicative of evolution of MVB's into residual bodies, and also of fusions among MVB's and other lysosomes were regularly encountered in the myoid regions. We have not made a detailed study of these structures.

DISCUSSION
Receptor Synaptic Activity

In light of the work on neurons outlined in the introductory paragraph, our finding that HRP uptake into rod and cone synaptic vesicles is decreased with light strongly suggests that frog photoreceptors are active in neurotransmission in the dark and less active in the light. This is in agreement with a substantial body of indirect physiological evidence which has been interpreted as suggesting that vertebrate photoreceptors release a depolarizing transmitter in the dark and that this release is diminished by light (12, 33, 55, 57).

The differences in levels of HRP uptake into rods and cones under various illumination conditions suggest that steady light affects rod synaptic activity to a much greater extent than cone synaptic activity. Evidently, moderate light intensities reduce transmitter release from rods to a low level while cones continue to release transmitter. This is consistent with an extensive body of physiological and psychophysical evidence that the rod response "saturates" at relatively low light intensities (1, 13, 18, 29, 44, 56) while cones continue to respond at relatively high intensities (2, 3, 4, 6, 29, 44).

Both our findings and the indirect physiological data indicate that: (a) rods recover their "dark" level of synaptic activity after moderate light intensities that bleach very little rod pigment (19, 30, 60); (b) cones, which, unlike rods, can regenerate their pigment in the absence of the pigment epithelium (17), recover their dark level of synaptic activity after full intensity light exposures that bleach over 90% of the cone pigment (4, 28); (c) rods release transmitter at intermediate rates at intermediate light intensities (44, 56); and (d) choline reduces receptor synaptic activity (7).

The many physiological parallels with our findings make it unlikely that our results are merely an artifact of the presence of HRP or of some nonspecific damage. Furthermore, our extracellular measurements of aspartate-isolated mass receptor potentials and ERG b-waves suggest that photoreceptors are not grossly affected by the presence of HRP. Of course, lack of any gross physiological effect of HRP as measured by these methods does not rule out some HRP effect at the single cell level. Intracellular recording is obviously needed to fully evaluate possible HRP effects. Similarly, we cannot rule out subtle effects of such factors as the absence of the pigment epithelium. As mentioned above, the obvious effect of this is the inability of rods to regenerate bleached pigment.

The rods do not recover their dark level of synaptic activity when kept in the dark for up to 60 min after full light intensity exposure which bleaches considerable amounts of rod pigment. Apparently, the permanent loss of pigment (17) is associated with a permanent loss, or very slow
FIGURE 8 The frequency distribution of HRP uptake levels into rod and cone terminals in retinas maintained with HRP in the dark for 60 min (a) or in full intensity light for 60 min (b). Bar heights represent averages from four repeat experiments. n per illumination condition per experiment was 20–30 terminals.

TABLE II

| Rod Recovery |
|--------------|
| Rod          | Average | SD  |
| Dark, dark   | 8.6     | 1.3 |
| Light, light | 1.4     | 1.4 |
| Light, dark  | 7.3     | 3.2 |

The proportion of the synaptic vesicles labeled with HRP in a recovery preparation treated with an initial −3.3 light exposure and then placed in the dark (light, dark) compared to labeling in companion preparations maintained only in the dark or in −3.3 light. n per data point is 20–30 terminals. Each retina was pretreated for 40 min in the absence of HRP in either dark or −3.3 light. Then in the presence of HRP, one retina was placed back in the dark, one placed back into the light, and the third, initially exposed to the light, was now placed in the dark.

recovery, of rod synaptic activity. This is consistent with the permanent decrease in sodium conductance observed in illuminated isolated frog outer segments (35) and the permanent loss in rod responsiveness in isolated retinas (14, 19, 30, 60) after extensive pigment bleaching.

Intracellular recording from eye-cup preparations of skate (11) and fish (41) after flash bleaches shows that the horizontal cells return to their dark membrane potential before pigment regeneration is completed. The interpretation can be drawn from these studies that rod synaptic activity, which presumably controls the membrane potential of horizontal cells, recovers quickly after extensive bleaches. The apparent discrepancy with our findings may be due to differences in the type of retinal preparations (eye-cup vs. isolated retina) or species differences. Thus, intracellular recordings from rods in isolated retinas of axolotl (19)
TABLE III

Recovery after Bleach

|        | Rod          | Cone        |
|--------|--------------|-------------|
|        | Average     | SD          | Average | SD          |
| A      |             |             |         |             |
| Dark   | 8.2         | 1.7         | 7.2     | 2.0         |
| Bleach, dark | 1.5     | 1.5         | 7.8     | 1.3         |
| B      |             |             |         |             |
| Dark   | 6.9         | 1.9         | 6.4     | 2.2         |
| Bleach, dark | 1.3     | 1.3         | 6.7     | 1.4         |
| Light, dark | 6.7      | 1.3         | 6.2     | 1.5         |

The proportion of synaptic vesicles labeled with HRP in preparations treated with an initial full intensity light exposure (bleaches 90% of the pigment) for 5 min compared to labeling in companion preparations treated in the dark. n per point per experiment is 20-30 terminals.

(A) The “dark” retina was exposed to HRP for 45 min. The companion retina was exposed to full intensity light for 5 min (“bleach”) and then allowed to dark adapt for 15 min, all in the absence of HRP. With HRP present, the retina was then kept in the dark for an additional 45 min. (B) The dark retina was exposed to HRP for 45 min. The other two retinas were exposed for 5 min to full intensity light (“bleach”) or to -3.3 light with HRP present and then, with HRP still present, they were placed in the dark for 40 min.

show that after pigment bleaches the cells remain relatively hyperpolarized for long periods. Additional work on the effects of pigment bleaching and regeneration on receptor membrane potential and synaptic activity for frog and other vertebrate retinas is needed to clarify the situation.

Fate of Vesicles

RECYCLING: The regions adjacent to the synaptic ribbons are the presumed sites of transmitter release from vertebrate photoreceptor terminals (10, 45). Thus, the fact that labeled vesicles accumulate along the ribbons suggests that endocytically formed synaptic vesicles within photoreceptor terminals may be reused for neurotransmission. Apparently, such vesicles accumulate at the ribbon at random, in the sense that, over the time periods we studied, HRP-labeled vesicles were neither markedly excluded nor notably concentrated along the ribbons, when compared with nonlabeled vesicles. Obviously, we know nothing of the transmitter content of the labeled vesicles, but these results might imply similar “randomness” in utilization of vesicles for neurotransmission (see also Fig. 12).

The reuse of endocytically formed vesicles for neurotransmission has been suggested from work on neuromuscular junctions (22, 31, 61, 62) and other neurons (54). It has also been proposed that structures such as coated vesicles and cisternae act as intermediate structures in the processing of retrieved membrane for possible reuse as synaptic vesicles (21, 22, 39). This is a matter of current dispute (see reference 31; the matter was discussed extensively at the 1975 Cold Spring Harbor Symposium on the Synapse). Coated vesicles labeled with HRP were seen too infrequently in our preparations to permit us to make any quantitative statements regarding their relationship to HRP uptake into other membranous structures. As for cisternae, we do see fairly large vacuoles that can accumulate HRP, but these arise after prolonged incubation and may be artifactual. We do not see the sorts of large, elongate sacs described in frog neuromuscular preparations (22). However, some small vacuoles, sacs, or short tubules were observed to accumulate HRP in the synapses at all times studied. These structures appear to increase

TABLE IV

Effects of Full Intensity Light on HRP Uptake

|        | Rod          | Cone        |
|--------|--------------|-------------|
|        | Average     | SD          | Average | SD          |
| Dark (4) | 10.8       | 2.0         | 10.1    | 1.8         |
| Light (4) | 1.2      | 0.3         | 5.4     | 2.0         |
| Light, dark (2) | 1.0 | 9.9    | 0.8 |             |

The proportion of the vesicle population labeled with HRP for retinal preparations exposed with HRP in the dark or full intensity light for 60 min. The number in parentheses is the number of experiments. On two occasions, a third retina was included in the experiment. This recovery retina was first placed in the absence of HRP in full intensity light for 60 min. Then, in the presence of HRP, it was placed in the dark for an additional 60 min. n per point per experiment is 20-30 terminals.

TABLE V

Effect of Choline

|        | Rod          | Cone        |
|--------|--------------|-------------|
|        | Average     | SD          | Average | SD          |
| Normal | 10.1        | 1.2         | 9.8     | 1.5         |
| Choline | 5.1       | 1.6         | 6.5     | 1.1         |

The proportion of the vesicle population labeled with HRP in retinal preparations exposed to HRP in the dark for 60 min with normal Ringer's or choline-Ringer's. Data are averages from two separate experiments. n per point per experiment was 20-30 terminals.
in frequency with increasing time in HRP in the dark, and it is possible that some participate in vesicle recycling (26).

**Degradation**: MVB's have been implicated in the degradation of membrane participating in endocytosis (see reference 24 for review and discussion of pertinent mechanisms). Our observations on the kinetics of MVB labeling in the
terminals, axons, and myoid regions are consistent with the view put forth for neurons (26, 54) that some of the membrane endocytically retrieved after transmitter release becomes incorporated into multivesicular bodies that eventually are transported in retrograde fashion (36, 37) to the perikaryon (in our case, the myoid region). In the myoid region, the MVB's probably acquire their acid hydrolases since it is here that acid phosphatase is demonstrable in Golgi-associated sacs or tubules and in numerous lysosomes, including MVB's (48).

Several "special" factors complicate quantification of the labeling of structures such as MVB's. These, along with the usual problems in unambiguously identifying early stages in MVB formation or in recognizing small MVB's, probably contribute to the variability noted in the comparisons of light- and dark-treated preparations. In particular, a given MVB in a photoreceptor terminal probably receives contributions of label from many endocytic vesicles, not all of which need be related to neurotransmission, and MVB's may also acquire tracer from structures such as endocytically derived tubules and perhaps through initial direct connections to the cell surface (24). Thus, for example, while one might expect the quantities of HRP that accumulate in individual MVB's to vary with different levels of synaptic uptake, the number of MVB's labeled might show less striking variations. One cannot reliably estimate quantities of HRP per body with microscope approaches of the type we used. We would also need more information than we have about relative rates of endocytic vesicle formation and fusion with MVB's, about MVB migration to the myoid region, and about the rates of transformation of MVB's into residual bodies in order to make adequate predictions about the details of MVB labeling.

In connection with the matters under discussion, it is interesting that autoradiographic and biochemical evidence suggests a rapid movement of lipids made in the myoid region into the terminals (5). This, in part, may reflect the steady-state replacement of vesicles that are degraded. The mechanisms of such replacement are considered elsewhere (26).

**HRP Uptake as a Measure of Synaptic Activity**

Our findings suggest that tracer uptake into synaptic vesicles and other membranous structures may be useful for monitoring synaptic activities in many systems. As with the present material, this may sometimes be possible even by light microscopy. One can also imagine use of fluorescent proteins or other tracers directly applicable to

**FIGURES**

9-12 Synaptic ribbons (arrows) from rod terminals in retinas exposed to HRP in the dark for 15 (Fig. 9), 30 (Fig. 10), 60 (Fig. 11), and 120 min (Fig. 12). Note the increase in the proportion of labeled vesicles at the ribbon with time. The configurations at the arrowheads may conceivably reflect exocytosis of vesicle contents, perhaps occurring during fixation (23) and releasing a vesicle-sized globule of HRP-containing material. However, such configurations were observed too infrequently for adequate analysis. We are made especially cautious in our evaluations of them by the fact that there is material of relatively high intrinsic electron density associated with the pre- and postsynaptic plasma membranes near ribbons (10). There is thus a risk of tangential sections of membranes producing misleading images, although in our experience most such sections produce larger and less electron-dense areas than the electron dense globules in Figs. 9 and 11, and Fig. 12 cannot be explained in this way (nor does the Fig. 12 configuration resemble the usual endocytic configurations, in which tracer very rarely fills the interior of forming vesicles). The bars represent 0.2 μm; × 49,000 (Fig. 9); × 56,000 (Fig. 10); × 43,000 (Fig. 11); and × 50,000 (Fig. 12).

**FIGURE 13** Portion of a rod axon (C) and terminal (T) containing several labeled MVB's (arrows). The retina was exposed to HRP for 60 min in the dark. The bar represents 1 μm; × 25,000.

**FIGURE 14** An HRP-labeled MVB (adjacent to M) in a rod terminal from a retina maintained in HRP in the dark for 30 min. R indicates a synaptic ribbon; E indicates extracellular space. The bar represents 0.5 μm; × 53,000.

**FIGURE 15** MVB's (arrows) labeled with HRP in a cone myoid region from a retina maintained in the dark for 60 min. The bar represents 0.5 μm; × 42,000.
living systems. It should be kept clearly in mind, however, that the precise quantitative relationships between the number of vesicles or other membranous structures containing HRP and the number of transmitter "quanta" released is not clear. For example, we do not know the relative timing of exocytic events and associated endocytic ones. There is evidence that endocytosis can sometimes lag behind exocytosis (22, 25, 31). We also lack crucial data on the "efficiency" of tracer uptake; perhaps a proportion of the endocytic vesicles that form lack detectable levels of label. Furthermore, compensatory endocytosis and exocytosis may occur at different sites along the surface (21, 22), and vesicle reuse or degradation can also complicate the quantitative picture. Clearly, there is still need for circumspection in interpretation.

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REFERENCES

1. AGUILAR, M., and W. A. STILES. 1954. Saturation of the rod mechanism of the retina at high levels of stimulation. Opt. Acta. 1:59-65.

2. ALPERN, M., W. A. H. RUSHTON, and S. TORIL. 1970. Signals from cones. J. Physiol. (Lond.). 207:463-475.

3. BACKSTRON, A. C., and T. REUTER. 1975. Receptive field organization of ganglion cells in the frog retina; contributions from cones, green rods and red rods. J. Physiol. Lond. 246:79-107.

4. BAYLOR, D. A., and A. L. HODGKINS. 1974. Changes in time scale and sensitivity in turtle photoreceptors. J. Physiol. Lond. 242:729-758.

5. BIBB, C., and R. W. YOUNG. 1974. Renewal of glycerol in the visual cells and pigment epithelium of the frog retina. J. Cell Biol. 62:378-389.

6. BOVTON, R. M., and D. N. WHITEN. 1970. Visual adaptation in monkey cones; recording of late receptor potentials. Science (Wash. D. C.). 170:1423-1426.

7. BROWN, J. E., and L. H. PINTO. 1974. Ionic mechanism for the photo-receptor potential of the retina Bufo Marinus. J. Physiol. Lond. 236:575-591.

8. CAJAL, S. RAMÓN-Y. 1972. The structure of the retina. Charles C Thomas. Publisher. Springfield, Ill.

9. CERVETTO, L., and E. MACNICHOLS. 1972. Inactivation of horizontal cells in turtle retina by glutamate and aspartate. Science (Wash. D. C.). 178:767-768.

10. DOWLING, J. E. 1974. Synaptic arrangements in vertebrate retina: the photoceptor synapse. In Synaptic transmission and Neuronal Interactions. M. V. L. Bennett, editor. Raven Press, New York. 87-103.

11. DOWLING, J. E., and H. RIPPS. 1971. S-potentials in the skate retina: intracellular recordings during light and dark adaptation. J. Gen. Physiol. 58:163-189.

12. DOWLING, J. E., and H. RIPPS. 1973. Effect of magnesium on horizontal cell activity in the skate retina. Nature (Lond.). 242:101-103.

13. FAI, G. L., and J. E. DOWLING. 1973. Intracellular recordings from single rods and cones in mud-
puppy retina. *Science (Wash. D. C.).* 180:1178-1180.

14. **FRANK, R. N.** 1971. Properties of “neural” adaptation in components of the frog electroretinogram. *Vision Res.* 11:1113-1123.

15. **FRIEND, D. S.,** and **M. G. FARQUHAR.** 1967. Function of coated vesicles during protein absorption in the rat vas deferens. *J. Cell Biol.* 35:357-376.

16. **FURUKAWA, T.,** and **I. HANAWA.** 1955. Effects of some common cations on the electroretinogram of the toad. *Jpn. J. Physiol.* 5:289-300.

17. **GOLDSTEIN, E. B.** 1967. Early receptor potential of the isolated frog retina. *Vision Res.* 7:837-845.

18. **GORDON, J., and D. HOOD.** 1976. Anatomy and physiology of the frog retina. In: The Amphibian Visual System: A Multidisciplinary Approach. Academic Press, Inc., New York. In press.

19. **GRABOWSKI, S. R., L. H. PINTO, and W. L. PAK.** 1975. Intracellular recordings of rod responses during dark-adaptation. *J. Physiol. Lond.* 247:363-391.

20. **GRAHAM, R. C.,** and **M. J. KARNOVSKY.** 1966. The early stages of absorption of injected HRP in the proximal tubules of mouse kidney. *J. Histochem. Cytochem.* 14:291-302.

21. **GRAY, E. G.,** and **H. L. PEASE.** 1972. On understanding the organization of the retinal receptor synapse. *Brain Res.* 35:1-15.

22. **HEUSER, J. E., and T. S. REESE.** 1974. Morphology of vesicle discharge and their reformation at the frog neuromuscular junction. In *Synaptic Transmission and Neuronal Interactions*. M. V. L. Bennett, editor. Raven Press, New York. 59-77.

23. **HEUSER, J. E., S. REESE,** and **D. M. LANDIS.** 1974. Functional changes in frog neuromuscular junctions studied with freeze-fracture. *J. Neurocytol.* 3:109-131.

24. **HOLTZMAN, E.** 1976. *Lysosemes: a survey.* Cell Biology Monograph Series. Springer-Verlag, Vienna.

25. **HOLTZMAN, E., A. F. FREEMAN,** and **L. A. KASHNER.** 1971. Stimulation dependent alterations in peroxidase uptake at lobster neuromuscular junctions. *Science (Wash. D.C.).* 173:733-736.

26. **HOLTZMAN, E., S. SCHACHER,** and **J. EVANS.** Membrane cycling and degradation in gland cells and neurons. In Membrane Assembly and Turnover. Cell Surface Reviews. G. Poste and G. L. Nicholson, editors. Noord-Hollandsche Uitg., Mij., Amsterdam. Vol. 4. In press.

27. **HOLTZMAN, E., S. TEICHERG, A. H. ABBREHAMS, E. CITKOWITZ, S. M. CRAIN, N. KAWAI,** and **E. R. PETTERSON.** 1973. Notes on synaptic vesicles and related structures, endoplasmic reticulum, lysosomes and peroxisomes in nervous tissue and the adrenal medulla. *J. Histochem. Cytochem.* 21:349-385.

28. **HOOD, D. C.,** and **P. A. HOCK.** 1973. Recovery of cone receptor activity in the frog’s isolated retina. *Vision Res.* 13:1943-1951.

29. **HOOD, D. C.,** and **P. A. HOCK.** 1975. Light adaptation of the receptors; increment threshold functions for the frog’s rods and cones. *Vision Res.* 15:545-553.

30. **HOOD, D. C., P. A. HOCK,** and **B. G. GROVER.** 1973. Dark adaptation of the frog’s rods. *Vision Res.* 13:1953-1963.

31. **HURLBUT, W. P.,** and **B. CECARELLI.** 1974. Transmitter release and recycling of synaptic vesicle membrane at the neuromuscular junction. In *Advances in Cytopharmacology*. Vol. 2. B. Cecarelli, F. Clementi, and J. Meldolesi, editors. Raven Press, New York. 141-154.

32. **JORGENSEN, O. S.,** and **E. T. MELLERUP.** 1974. Endocytic formation of rat brain synaptic vesicles. *Nature (Lond.).* 249:770-771.

33. **KANEO, A.,** and **H. SHIMAZAKI.** 1975. Effects of external ions on the synaptic transmission from photoreceptors to horizontal cells in the carp retina. *J. Physiol. Lond.* 252:509-522.

34. **KARNOVSKY, M. J.** 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* 27(2):137-138 a. (Abstr.)

35. **KORENBROT, J. I.,** and **R. A. CONE.** 1972. Dark ionic flux and the effects of light in isolated rod outer segments. *J. Gen. Physiol.* 60:20-45.

36. **KRISTENSSON, K., Y. OLSSON,** and **J. SIÖSTRAND.** 1971. Axonal uptake and retrograde transport of exogenous protein in the hypoglossal nerve. *Brain Res.* 32:399-406.

37. **LA VAIL, M.,** and **J. LA VAIL.** 1974. Organelles involved in retrograde axonal transport in chick ganglion cells. *J. Comp. Neurol.* 157:303-358.

38. **LUF, J. M.** 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* 2:409-414.

39. **MODELL, P. G., S. M. HIGHSTEIN,** and **M. V. L. BENNETT.** 1973. Depletion of presynaptic vesicles accompanies rapid stimulation at a central synapse. Abstracts, Third Annual Meeting of the Society for Neurosciences. San Diego, Calif.

40. **NAGASAWA, J., W. W. DOUGLAS,** and **R. SCHULTZ.** 1971. Micropinocytic origin of coated and smooth microvesicles in neurosecretory terminals of posterior pituitary glands demonstrated by incorporation of HRP. *Nature (Lond.).* 232:341-342.

41. **NAKA, K. I.,** and **W. A. H. RUSHTON.** 1968. S-potential and dark adaptation in fish. *J. Physiol. (Lond.).* 194:259-269.

42. **NEGISHI, K.,** and **K. SUGAWARA.** 1973. Evidence for the anoxia sensitivity of the synaptic region at the outer plexiform layer in the fish retina. *Vision Res.* 13:983-987.

43. **NILSSON, S. E. G.** 1964. An electron microscopic classification of the retinal receptors of the leopard.
frog. J. Ultrastruct. Res. 10:390-416.
44. Normann, R. A., and F. S. Werblin. 1974. Control of retinal sensitivity. I. Light and dark adaptation of vertebrate rods and cones. J. Gen. Physiol. 63:37-61.
45. Ravina, E., and N. B. Gilula. 1975. Intramembrane organization of specialized contacts in the outer plexiform layer of the retina. A freeze-fracture study in monkeys and rabbits. J. Cell Biol. 65:192-222.
46. Rips, H., M. Shaked, and E. D. MacDonald. 1974. Turnover of synaptic vesicles in photoreceptor terminals of the skate. Biol. Bull. (Woods Hole). 147:495.
47. Saratini, D. D., K. Bensch, and R. J. Barnett. 1963. Cytochemistry and electron microscopy: the preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. 171:19-58.
48. Schacher, S., E. Holtzman, and T. Ebrey. 1973. Cytochemical studies of peroxidase uptake and other features of frog retinal photoreceptor cells. J. Histochem. Cytochem. 21:419.
49. Schacher, S., E. Holtzman, and D. Hood. 1973. Cytochemical studies of frog retinal photoreceptor cells. Abstracts, Third Annual Meeting of the Society for Neurosciences. 107.
50. Schacher, S., E. Holtzman, and D. Hood. 1974. Uptake of horseradish peroxidase by frog photoreceptor synapses in the dark and the light. Nature (Lond.). 249:261-263.
51. Schacher, S., E. Holtzman, and D. Hood. 1975. Synaptic activity of frog photoreceptors. Association of Research in Vision Ophthalmology Conference. Sarasota, Fla. 75.
52. Schacher, S., E. Holtzman, and D. Hood. 1975. HRP uptake measures synaptic activity of vertebrate photoreceptors. Abstracts, Fifth Annual Meeting of the Society for Neurosciences. New York. 113.
53. Sillman, A. J., H. Ito, and T. Tomita. 1969. Studies on the mass receptor potential of the isolated frog retina. II. On the basis of the ionic mechanism. Vision Res. 9:1443-1451.
54. Teichberg, S., E. Holtzman, S. M. Crain, and E. R. Peterson. 1975. Circulation and turnover of synaptic vesicle membrane in cultured fetal mammalian spinal cord neurons. J. Cell Biol. 67:215-230.
55. Toyoda, J. 1973. Membrane resistance changes underlying the bipolar cell response in the carp retina. Vision Res. 13:283-294.
56. Toyoda, J., H. Hashimoto, H. Anno, and T. Tomita. 1970. The rod response in the frog as studied by intracellular recording. Vision Res. 10:1093-1100.
57. Trifonov, Yu. A. 1968. Study of synaptic transmission between photoreceptors and horizontal cells by electric stimulation of the retina. Biophysics (Engl. Transl. Biofise.). 13:948-957.
58. Turner, P. T., and A. B. Harris. 1974. Ultrastructure of exogenous peroxidase in cerebral cortex. Brain Res. 74:305-326.
59. Venable, J. H., and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25:407-408.
60. Weinstein, G. W., R. R. Horson, and J. E. Dowling. 1967. Light and dark adaptation in the isolated rat retina. Nature (Lond.). 215:134-138.
61. Zimmerman, H., and V. P. Whittaker. 1974. Effect of electrical stimulation on the yield and composition of synaptic vesicles from the cholinergic synapses of the electric organ of Torpedo, combined biochemical, electrophysiological and morphological study. J. Neurochem. 22:435-450.
62. Zimmerman, H., and V. P. Whittaker. 1974. Different recovery rates of electrophysiological, biochemical and morphological parameters in cholinergic synapses of the Torpedo electric organ after stimulation. J. Neurochem. 22:1109-1114.