

# ELECTRON MICROSCOPE AUTORADIOGRAPHIC DETECTION OF SITES OF PROTEIN SYNTHESIS IN THE RABBIT RETINA MÜLLER CELLS

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## ABSTRACT

Rabbit retinas were incubated in medium containing 500  $\mu$ Ci of [ $^3$ H]leucine for 3 min, and transferred to medium without isotope for another 7, 17, 37, 57, and 117 min. Retinal pieces were fixed in paraformaldehyde and osmium tetroxide and embedded in Epon. Thin sections were autoradiographed with Ilford L4 emulsion, and a quantitative study of silver grain distribution per Müller cell portion, and per Müller cell organelle, was carried out. Grain density per unit area was high over the middle cell portion at each incubation interval. Silver grains were numerous over background cytoplasm (which comprised free ribosomes) but their percentage was constant at all times and their relative concentration low. Silver grains were numerous and highly concentrated, at pulse incubation, over the rough endoplasmic reticulum (RER) and then decreased sharply, but this decline coincided with an increase over the Golgi complex, peaking at 20 min. Another peak appeared over the cell periphery at 60 min. These findings suggest the simultaneous synthesis of two types of proteins in Müller cells: structural proteins in background cytoplasm and proteins of secretory type in the RER.

## INTRODUCTION

Müller cells exhibit different ultrastructural features along their length across the retina. In the rabbit, three portions with distinct subcellular and cytochemical organization can be recognized in the Müller cell, which may correspond to a specialization of metabolic functions in each of them (12). The inner portion, for example, is very rich in glycogen particles and in glucose-6-phosphatase-positive, smooth-walled vesicles. It appears to be the main source for the carbohydrate used in anaerobic glycolysis by the inner retina (10, 11).

The middle portion of the cell, which is situated in the inner nuclear layer, contains a much smaller amount of those elements, but, on the other hand, it is the site of all the ribosomes, the ergastoplasm,

and the Golgi complex of the cell (12). This portion should thus be particularly engaged in protein synthesis.

It was therefore decided to use electron microscope autoradiography to detect the sites of protein synthesis and migration in Müller cells. The results showed not only that the middle portion was much more heavily labeled than the other two portions, but also that two kinds of proteins with different kinetics, one sedentary, the other probably of secretory type, were formed in it. This paper presents and analyzes such data, which may also be of interest with respect to our knowledge of glial protein synthesis in general, considering that Müller cells are a special type of astrocyte.

## MATERIALS AND METHODS

Two adult male rabbits ~1,500 g in weight were anesthetized with urethane (900 mg/kg body weight) injected into the ear vein. The eyes were rapidly removed and placed in a Petri dish containing the medium of Ames and Hastings (1) well aerated with a mixture of 5% CO<sub>2</sub> and 95% O<sub>2</sub>, at 0°C. The eyeballs were sectioned through the equatorial plane, and the retinas were detached and divided into several pieces; these pieces did not include the horizontal bands on both sides of the papilla which are the only parts of the rabbit retina that are provided with blood vessels. All operations were performed in 1-2 min with the tissues under the medium. The pieces were incubated in shaken flasks containing 1 ml of medium plus 500  $\mu$ Ci of L-[4,5-<sup>3</sup>H]leucine (sp act ~30,000 mCi/mmol, Radiochemical Centre, Amersham, England), for 3 min, at 37°C. Some fragments were fixed, while the remaining pieces were further incubated in fresh medium deprived of [<sup>3</sup>H]leucine but containing 2 mM/liter of non-radioactive leucine for another 7, 17, 37, 57, and 117 min, respectively, before fixation.

Tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer with 0.3% nonradioactive leucine, pH 7.3, for 3 h at room temperature (fixative was changed ten times); rinsed once in the same buffer with 10% sucrose, 0.005% CaCl<sub>2</sub>, and 0.13% leucine; and rinsed again in three changes of the same buffer without leucine (2). They were postfixed in 1% osmium tetroxide in the same buffer for 2 h at 4°C, and embedded in Epon. 1-2  $\mu$ m semithin sections were stained with periodic acid-Schiff-hematoxylin (PAS + H) and autoradiographed with stripping film Kodak AR10 (Eastman Kodak Co., Rochester, N. Y.). Ultrathin sections were autoradiographed with Ilford L4 emulsion diluted 1:4 in distilled water (7). After uniform exposures of 35 or 48 days, thin

sections were stained with uranyl acetate for 30 min and lead citrate for 10 min.

For determination of silver grain densities per unit cytoplasm area, sections with identical exposures were photographed at  $\times 5,000$  and the prints enlarged  $\times 3$ . The total cytoplasmic areas of the inner, middle, and outer portions of Müller cells visible in each micrograph were cut out and weighed in order to measure them; the same was done for perikarya of bipolar cells. Nuclei were, in each case, not included in the areas. Silver grains overlying each of those areas were counted in each print, and the radioactivity was expressed as silver grain density per 100  $\mu$ m<sup>2</sup> of cytoplasm. Density values for the two experiments were shown to vary similarly with time by an analysis of variance ( $F$  with  $P \approx 0.05$ ); therefore the average of the values from the two experiments was used as the final result (Fig. 5).

Silver grains were assigned to the various cytoplasmic structures in the cell middle portion according to the method of Whur et al. (16), by placing circles with 2,250 Å radius over the center of silver grains and recording the structures falling within the circles. The data were expressed as percentage of grains per structure. Since an analysis of variance of these values (after angular transformation) also showed the percentage of grains per structure to vary similarly with time in the two experiments (mean square of interaction *structure*  $\times$  *experiment* was 18.3 and the rest was 21.5), the average of the values from both experiments was again used as a final result (Table I). 500 identical circles for each experiment were placed at random over the middle portion cytoplasm (two micrographs were used for each time in each experiment and the values were pooled). The number of structures hit by these random circles or by random points which were the centers of the circles, was also recorded. The number

TABLE I  
Percentages of Silver Grains over Cell Components  
(Chase Incubation after a 3 min Pulse)

	3 min	10 min	20 min	40 min	60 min	120 min	Random points	Random circles
RER	17.5	5.3	3.7	1.5	1.0	2.9	4.7	5.1
Golgi complex	2.0	8.0	18.9	9.1	6.4	7.0	5.7	6.3
Background	78.5	85.5	75.0	84.0	83.4	84.0	86.7	87.1
cytoplasm								
Cell periphery	2.0	1.2	2.4	5.4	9.2	6.1	2.9	1.5
No. of grains counted	655.5	594	802	633.5	480.5	363		

The data represent the average of the two experiments (total number of grains counted 3,528.5).

of points over each structure served to estimate its percentage area. Relative grain concentrations were expressed by the ratio:

$$\frac{\text{Percent of the grains over a structure}}{\text{percent of total area occupied by a structure}}.$$

The following cytoplasmic structures of the middle portion were studied by this method: (a) *rough endoplasmic reticulum* (RER) (the ergastoplasmic profiles); (b) *Golgi complex* (cisternae and associated vesicles); (c) *cell periphery* (a 2,250 Å wide band to the inner side of the cell membrane); (d) *background cytoplasm* (all the background cytoplasm excluding those three structures; free ribosomes entered in this category).

## RESULTS

The Müller cell fine structure has been recently described in the rabbit (12). Some details should, however, be added with respect to the morphology of the protein-synthesizing structures. The cell RER is present only in the middle portion. It is composed of a number of ergastoplasm profiles, single or in stacks of two to four cisternae, dispersed throughout the cytoplasm (Figs. 1 and 2). Free ribosomes and polyribosomes are observed in the middle portion but not in the rest of the cell. The nuclear outer membrane is studded with ribosomes. The Golgi complex is located in the outer half of the middle portion and consists of a few stacks of curved cisternae with adjoining vesicles (Fig. 1). The Golgi stacks may be close together, or widely separated; they often are near RER cisternae.

This description is based on glutaraldehyde-fixed material. Paraformaldehyde fixation caused some dilation of the perinuclear and the ergastoplasmic cisternae, lower membrane contrast, and some breakage of membranes, but the general appearance was the same as with glutaraldehyde.

No appreciable ultrastructural alterations were observed in retinas incubated for periods up to 120 min. At this incubation time, there was a moderate shrinkage of nerve cell processes and a tendency for nuclei of rods and cones to be pycnotic. The hyaloplasm of Müller cells became somewhat lighter, but no other changes were apparent.

### Autoradiography

Light microscope autoradiographs showed different locations of silver grains at different incubation times. After pulse labeling, most silver grains

tended to be concentrated over the layer of rods and cones, the inner nuclear layer, and the ganglion cell layer (Fig. 3). After chase incubations, silver grains became more dispersed (Fig. 4).

Electron microscope autoradiographs showed autoradiographic reactions over both Müller and nerve cells at all incubation times; silver grains were more numerous over nerve cells. The background fog averaged 1.2 silver grains per 100  $\mu\text{m}^2$ .

In Müller cells, the middle portion always contained a much higher silver grain density than the outer or the inner portion (Fig. 5). The analysis of the distribution of silver grains per organelle in the cell middle portion showed a high percentage of grains over the background cytoplasm at all incubation times, but these values did not differ markedly from, and were lower than, random circle or random point values (Table I). On the other hand, the silver grain percentage over the RER was much higher than random values at pulse incubation (Table I and Fig. 6), and became very low after chase (Table I). A high value for the Golgi complex showed up at 20 min (Table I and Figs. 7 and 8). Percentages over the cell periphery were high at later times (Fig. 9), peaking at 60 min (Table I). Values obtained by dividing grain percentages by random point percentages, thus expressing relative grain concentration per organelle, made these patterns more clear (Fig. 10). The concentration was high over the RER at 3 min, then decreased sharply. A peak over the Golgi complex was apparent at 20 min. There was a high grain concentration over the cell periphery at 60 min when silver grain densities were low over the other two compartments. Values over background cytoplasm were low and constant throughout the several incubation times (Fig. 10).

In the inner and outer portions of Müller cells, the few silver grains were dispersed over all the cytoplasm, with no particular association with any type of organelle. No accumulation of silver grains was noted over the Müller cell microvilli or the inner limiting membrane.

## DISCUSSION

To avoid binding of free [ $^3\text{H}$ ]leucine to the incubated retina, paraformaldehyde fixation was used instead of glutaraldehyde (5, 14). Paraformaldehyde was slightly less satisfactory with regard to membrane fixation; however, it provided reasonable preservation of retinal ultra-

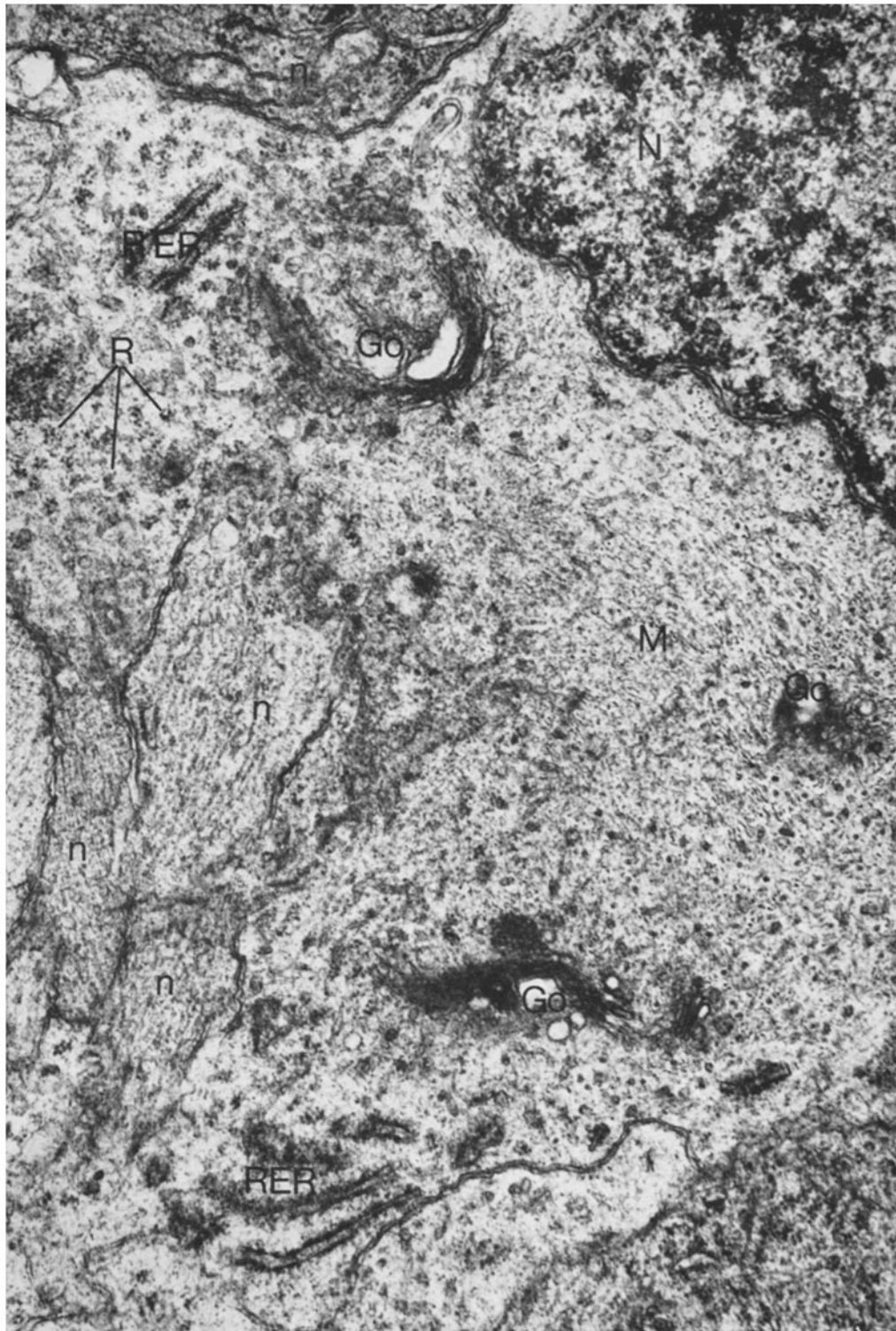


FIGURE 1 Electron micrograph of the middle portion of the rabbit Müller cell (*M*). The figure shows two stacks of RER cisternae (*RER*) and three stacks of the Golgi complex (*Go*). In the background cytoplasm, polyribosomes (*R*), and microfilaments. Glycogen particles are not visible owing to postfixation with uranyl acetate which extracted glycogen. *n*, nerve cell processes; *N*, Müller cell nucleus. Fixation, glutaraldehyde + osmium + uranyl acetate; staining, lead citrate.  $\times 25,600$ .



FIGURE 2 The picture shows a RER stack made up of three parallel cisternae in the Müller cell middle portion (M). Numerous cytoplasmic particles are either ribosome or glycogen beta particles; the specimen was not refixed with uranyl acetate. n, nerve cell processes. Fixation, glutaraldehyde + osmium; staining, lead citrate.  $\times 58,500$ .

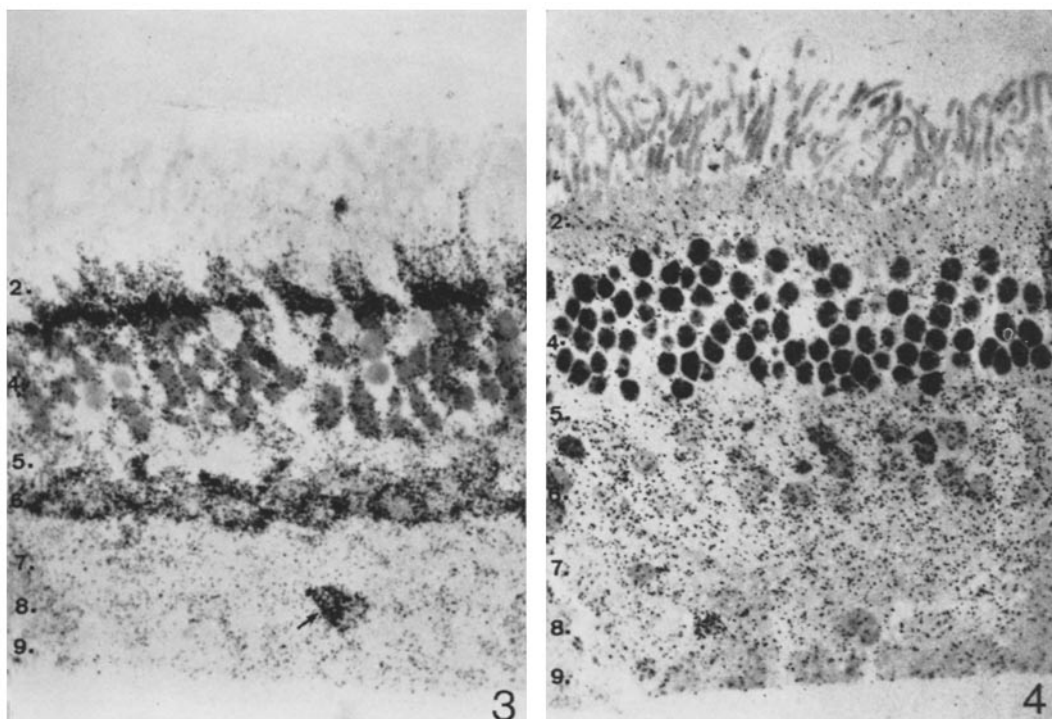
structure, including the Müller cell. Incubation in vitro, on the other hand, did not induce further morphological changes in this cell, while it permitted pulse labeling and subsequent chase in order to follow intracellular displacements of newly formed proteins.

The exclusive intracellular location of RER cisternae, free ribosomes, and the Golgi complex in the middle portion of Müller cells (12) was in itself highly suggestive that this middle portion was the predominant place for protein synthesis. Quantitative autoradiography confirmed this expectation by showing a much higher rate of synthesis in that portion than in the rest of the cell (Fig. 5). In addition, the analysis of the silver grain distribution per organelle, at different times, revealed the formation of proteins with distinct migration kinetics in different locations. Labeled proteins in the background cytoplasm persisted in constant amount and low concentration, as a plateau, across the successive chase incubation intervals (Table I and Fig. 10). This behavior, and

the fact that this compartment contained the free ribosomes of the cell, were taken as indicating that these proteins were structural or nonexportable proteins (9).

On the contrary, labeled proteins present at high concentration at 3 min in the RER fell abruptly in this organelle during subsequent chases, but, while this happened, a peak appeared at 20 min over the Golgi complex, and another was detected later on over the cell periphery (Fig. 10). This sequence of peaks was suggestive of the existence of proteins of secretory or exportable type (9) going successively through the three compartments (3).

Since the percentage and concentration of silver grains over the RER at 3 min, and over the Golgi complex at 20 min, were about the same, and the relative areas of the two organelles were also similar, it seemed that most of the proteins formed in the RER during the pulse might be present in the Golgi complex at 20 min. The further destination of these proteins was more difficult to analyze because of the absence of secre-



FIGURES 3 and 4 Light microscope autoradiographs of rabbit retinas incubated in Ames and Hastings's medium containing labeled leucine. Exposure, 26 days; staining, PAS + H.

FIGURE 3 After 3 min (pulse) incubation, the silver grains appear predominantly over the inner segment of the layer of rods and cones (numbered 2 at left), the inner nuclear layer (6) and over ganglion cells (layer 8; arrow).  $\times 528$ .

FIGURE 4 At 120 min incubation (3 min pulse + 117 min chase), the silver grains are diffusely distributed over the retina.  $\times 496$ .

tion granules in the Müller cell. An analogous situation was found by Zagury et al. (17) in immunoglobulin production by plasma cells. It was proposed that in plasma cells the Golgi-stored proteins could be released into the cell sap before crossing the plasma membrane in dispersed form (17). In Müller cells the Golgi complex was not far from the cell periphery, but no examples of fusion of Golgi and plasma membranes were ever observed. Hence, a release in dispersed form into the peripheral cytoplasm was also considered possible in this cell, and it was reasoned that by counting silver grains over a peripheral band having the width of the resolution boundary circles of Whur et al. (16), the majority of radioactive proteins present diffusely in cytoplasm close to the cell membrane could be detected. In fact, the

relative concentration of radioactivity in this region was very high at 60 min, suggesting release in dispersed form as a probable way of protein discharge from the Müller cell. It should, however, be remarked that the strong labeling of the immediately adjacent bipolar cells at all times precluded an analysis of the further destination of these proteins outside of Müller cells.

With regard to the small amount of labeled material observed at all incubation times in the extreme portions of the cell, it is not probable that this material consisted of proteins previously stored in the Golgi complex because it was not increased at late times (Fig. 5). Since the grain densities were, on the contrary, parallel to that present in the middle portion during the experiment (Fig. 5), it is rather likely that labeling in

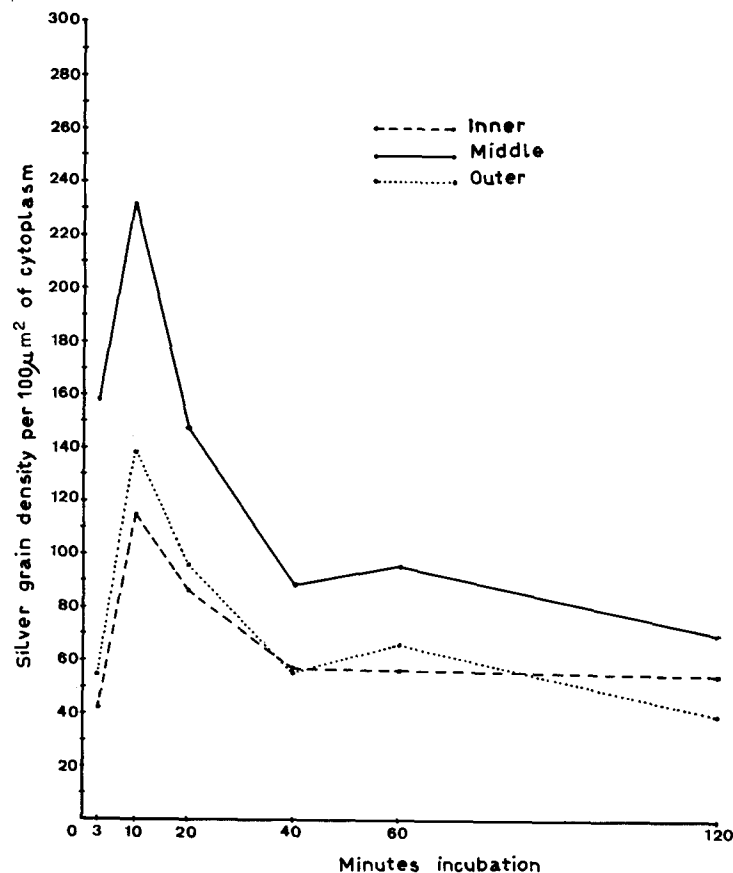


FIGURE 5 The graph shows the curves demonstrating the variation of silver grain density per unit cytoplasm area (determined by weighing) in electron microscope autoradiographs of the three Müller cell portions, with the incubation times. Each value is the mean of the two experiments. (Total number of grains counted, 7,910.)

the inner and outer portions was due to structural proteins that formed in the free ribosomes of the middle portion and then moved continuously to the cell ends to be used in renewal of local cell components.

Müller cells are rich in several enzyme activities (4, 8) and it is probable that most of the newly formed proteins of sedentary type here described are enzymatic. The nature and functions of the RER-synthesized, possibly exportable proteins should be completely different. As a matter of fact, Müller cells have many features in common with brain astrocytes (12, 15). In brain, protein synthesis takes place mainly in neurons, but it also occurs in glial cells; and there is one protein at least, S-100 (13), which is mostly

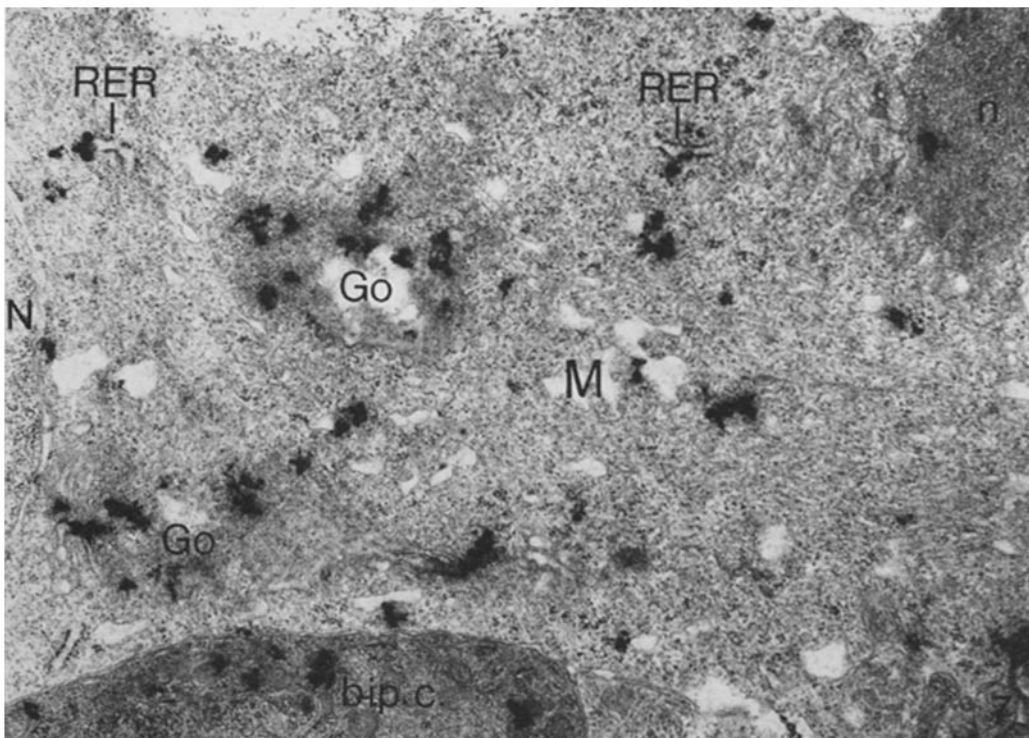
localized in glial cells, and another, 14-3-2, present in both cell types (13). It has also been suggested that RNA and proteins can be transferred from glial to nerve cells under stimulating conditions (6). It is thus possible that exportable proteins synthesized in the Müller cells can be similarly transferred to the retinal neurons.

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**FIGURE 6** Electron microscope autoradiograph of rabbit retina after 3 min (pulse) incubation. Most silver grains are located over the cisternae of the RER of the Müller cell middle portion (*M*). *N*, Müller cell nucleus. Fixation, glutaraldehyde + osmium; staining, uranyl acetate + lead citrate.  $\times 35,000$ .

**FIGURE 7** Low power autoradiograph at 20 min incubation showing many silver grains associated with the Golgi complex (*Go*) of the Müller cell (*M*) and a smaller number with the RER. *n*, nerve cell process; *bip. c.*, bipolar cell. Fixation and staining as for Fig. 6.  $\times 17,000$ .



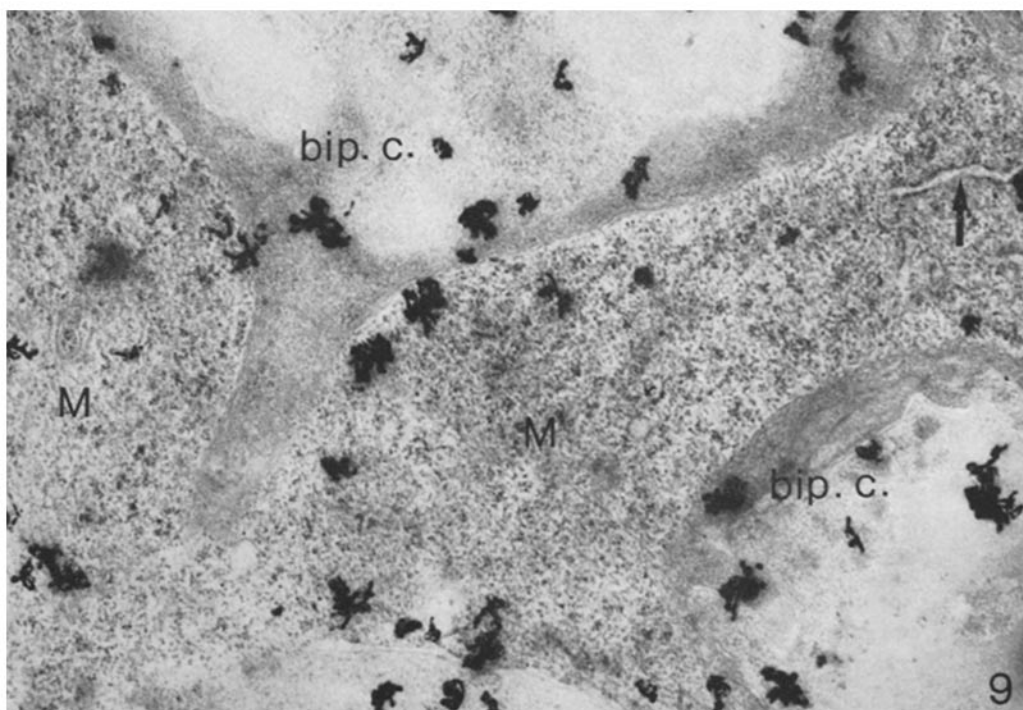
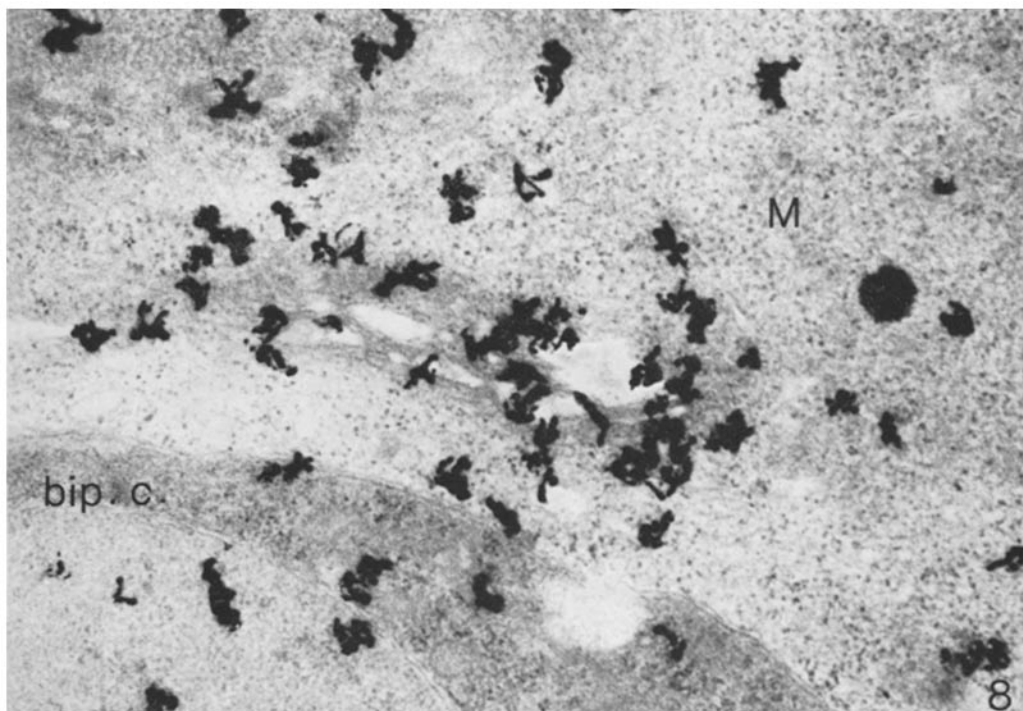


FIGURE 8 The picture shows that at 20 min incubation, the majority of silver grains are located over the Golgi complex. M, Müller cell; *bip. c.*, bipolar nerve cell. Fixation and staining as for Fig. 6.  $\times 27,000$ .

FIGURE 9 After 120 min of incubation, silver grains are numerous over the Müller cell (M) periphery. *bip. c.*, bipolar nerve cell; arrow, RER cisternae. Fixation and staining as for Fig. 6.  $\times 15,000$ .

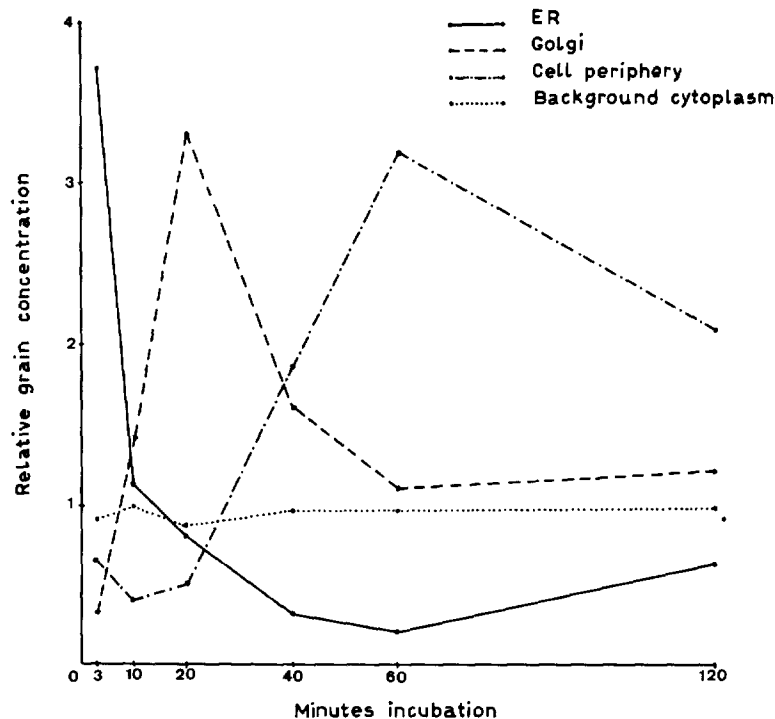


FIGURE 10 Graph showing relative concentration of the label (ratio of grain counts for a structure over the relative area occupied by the structure) (mean of two experiments). It is possible to observe changes in concentration suggesting transfer of the label from one structure to another.

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