ELECTRON MICROSCOPE

RADIOAUTOGRAPHIC STUDY OF GLYCOGEN

SYNTHESIS IN THE RABBIT RETINA

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ABSTRACT

Glycogen is present in the rabbit retina in monoparticulate form. Beta particles (~ 229 A) are abundant in Müller cell cytoplasm, particularly in its inner portion, decreasing in number outwards along the cell. They are slightly larger (~ 250 A) and much scarcer in neurons, though regularly present in the juxtanuclear Golgi region of ganglion cells. When the retina was incubated in a glucose-free medium, it was rapidly depleted of native glycogen. On further incubation in medium containing glucose-1H plus unlabeled glucose, glycogen reappeared in the form of beta particles of the same size and distribution as native ones, while radioautography revealed the appearance of amylase-labile radioactivity in the same locations. This newly formed glycogen was not associated with any particular organelle. The rate of synthesis, as judged from the amount of radioactivity, was high in the inner portion of Müller cells and declined uniformly toward the cell outer end, following a logarithmic gradient. The rate of synthesis was low in ganglion cells, at best approaching values in the outer portion of Müller cells. The concentration of glycogen in the inner portion of Müller cells is consistent with the view that it may be the source of glucose for the anaerobic glycolysis prevailing in the inner retina.

INTRODUCTION

The rabbit retina is exceptionally rich in glycogen (14). Since this tissue exhibits high rates of respiration and glycolysis (4, 5, 18, 19) but is probably poorly supplied with systemic glucose which must come mainly by diffusion from the choroid, glycogen may provide for immediate energy needs. Retinal glycogen has been shown by light microscopy and PA-Schiff staining to be abundant in the glial cells of Müller and to reaccumulate in those cells during incubation with glucose after previous depletion (14). It was concluded from such studies that glycogen storage occurred in Müller cells but not in neurons (14), so that the former would play the role of carbohydrate reservoirs for the whole retina.

In the course of an electron microscope study of the rabbit retina, we have partly confirmed such findings by observing a remarkable richness of glycogen particles in the Müller cell cytoplasm. In addition, we noted systematic differences in the density of glycogen particles within the cell, in such a way that they were always more numerous in the inner cell region, fewer in the middle perinuclear portion, and even fewer in the outer portion of the cell. This variation would agree with the presumable carbohydrate reservoir function of the Müller cell, since glycogen amounts would thus be higher in those retinal layers located farther from the choroid and, therefore, less well provided with systemic oxygen and glucose, in
which anaerobic glycolysis is assumed to prevail (16, 19). However, we have, on the other hand, observed that retinal neurons also contained a small amount of glycogen particles, and this would argue against the exclusive glial location of glycogen storage (14).

It was, therefore, decided to use electron microscope radioautography to identify the cellular sites of glycogen synthesis with accuracy, and to compare the rates of synthesis among them. To this purpose, rabbit retinas were incubated with radioactive glucose, and the amylase-labile radioactivity was estimated in electron microscope radioautographs in the three portions of Müller cells and of ganglion cells taken as an example of the retinal neuron.

MATERIALS AND METHODS

Three adult male rabbits, 1500 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 Hastings et al. (9) (K+, 110 mm; Ca++, 10 mm; Mg++, 10 mm; HCO3-, 40 mm; CI-, 130 mm; gassed with 95% O2 to 5% CO2 mixture giving a pH of 7.35 before incubation) at 37°C. The eyeballs were moved and placed in a Petri dish containing the medium and fixed. Then the cytoplasmic areas occupied by the innermost retinal layers. The number of grains in ten such random areas were counted on each section, and the mean background count measured outside the sections was deducted from the mean of the retinal measurements.

For electron microscopy, tissues were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hr at 4°C, kept in sucrose buffer overnight, and postfixed in 1% osmium tetroxide in veronal-acetate buffer at pH 7.3 for 2 hr. After fixation in glutaraldehyde and washing in sucrose buffer, some pieces were immersed in 1% alpha-amylase in phosphate buffer 0.01 M, pH 7.0 at 37°C, for 2 hr, or in buffer for the same period of time, washed again in sucrose buffer, and postosmicated. Embedding in Epon was carried out after dehydration in ascending alcohols. 1-2-µm sections were stained with PA-Schiff and radioautographed the same way as the paraffin sections. Ultrathin sections were radioautographed with Ilford L4 emulsion diluted one part to three parts of distilled water (12); for dipping the slides, the semi-automatic technique of Kopriwa was followed (11). After a uniform exposure of 60 days, the sections were stained with lead citrate (20) and examined in a Siemens Elmiskop I A. For determination of silver grain densities per unit area, negatives were photographed at X 5,000 and the prints enlarged X 3. Then the cytoplasmic areas occupied by the inner (bordering the vireous), the middle (perinuclear), and the outer (bordering the choroid) portions of Müller cells which were visible in each print were cut out and weighed in order to measure them. The same was done for perikarya of ganglion 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 the density of silver grains per 100 µ2 of cytoplasm. Electron micrographs of control and incubated pieces from experiments I and II, with final magnifications of 60,000, served to determine the concentration of glycogen particles per 1 µ2 of Müller cells. A translucent square area of 2 cm side was moved over the cell at random, the particles within 50 areas from each of the three Müller cell portions were counted, and the mean number was expressed per 1 µ2 area. The diameter of 100 glycogen particles in each of the three Müller cell portions from experiment II was measured on three photographic prints by using a...
Bausch & Lomb measuring magnifier (cat. 81-34-35). The diameters of 100 beta particles and 100 ribosomes were measured by the same procedure in ganglion cells from unincubated control pieces from the same experiment. The diameters of 100 of the ribosome-like granules, which were the only ones observed in adjacent sections stained with uranyl acetate, and in lead-stained sections from blocks treated with alpha-amylase, were also measured in each case. When glycogen particles or ribosomes were slightly oval rather than round, the larger diameter was the one measured.

RESULTS

Native Glycogen

In control retinas fixed before incubation, the PA-Schiff-positive, amylase-labile material occurred in the ganglion cell and optic nerve fiber layers as elongated masses interspersed among the ganglion cells and perpendicular to the vitreous surface (Fig. 1). More externally, finely dispersed material was present in the inner nuclear and both plexiform layers, and in the inner zone of the layer of rods and cones (Fig. 1). Among this material, slender profiles could be distinguished which prolonged the inner masses outwards (Fig. 1). The distribution was suggestive of a prevalent location in Müller cells, as pointed out by Kuwabara and Cogan (14), and this was confirmed by the electron micrographs. In fact, round beta particles which had strong lead affinity and which disappeared after alpha-amylase digestion were particularly numerous in Müller cell cytoplasm (Figs. 2-4). They were evenly distributed over the hyaloplasm without being clustered at any special site or associated with any particular organelle (Figs. 2-4). Their diameter was rather constant (Table I), but their density per unit area of cytoplasm varied appreciably among the three main portions of the cell (Table II), which differ strikingly among

![Figure 1](image_url)
Electron micrographs of the three Müller cell portions, from unincubated retinas. Fig. 2 shows the inner portion (facing the vitreous [V]) of two Müller cells (M1, M2). Numerous beta particles are evenly distributed throughout the cytoplasm which also contains a great amount of smooth-walled cisternae. Although in this picture the vesicles form a tight network at one place (arrow), this is not the rule since the vesicles often appear disseminated throughout the cell as in Fig. 9. Fig. 3 depicts the middle cell portion in the inner nuclear layer. At the left is the cell nucleus (N); in the middle of the picture is the cytoplasm (M) which contains beta particles which appear to be less numerous than those in the inner portion, some smooth cisternae, and a few ergastoplasmic profiles (arrows). At the upper right corner are nerve cell processes (n) of the bipolar layer neuropil. Fig. 4 shows the outer portion of the cell (M) with even fewer beta particles among microtubules over a lighter cytoplasmic background. Two portions of retinal rods (R1, R2), and nerve cell processes (n) of the neuropil of the outer nuclear layer are visible. Lead citrate. X 20,000.
TABLE I
Glycogen Particle Mean Diameters (in Angstroms) in the Three Müller Cell Portions

| Retinal pieces | Inner     | Middle    | Outer     |
|----------------|-----------|-----------|-----------|
| Unincubated control | 226.2 ± 1.8 | 227.1 ± 1.9 | 228.7 ± 2.1 |
| 30-min incubation    | 230.8 ± 1.9 | 228.8 ± 2.1 | 231.9 ± 1.7 |
| 60-min incubation    | 227.4 ± 2.1 | 228.6 ± 2.1 | 231.9 ± 2.1 |

Each value is the mean of 100 measurements ± se (standard error of the mean). P values among zones in each retinal piece, and among pieces in the same zone were >0.05.

TABLE II
Glycogen Particle Densities per μ² of Müller Cell Cytoplasm in Each Müller Cell Portion

| Experiment | Retinal pieces | Inner     | Middle    | Outer     |
|------------|----------------|-----------|-----------|-----------|
| I          | Control (unincub.) | 209.1 ± 4.5 | 181.8 ± 2.7 | 145.5 ± 4.5 |
|            | 30-min incub. | 100.0 ± 2.7 | 72.7 ± 2.7 | 45.4 ± 1.8 |
|            | 60-min incub. | 136.5 ± 3.6 | 100.0 ± 2.7 | 72.7 ± 2.7 |
| II         | Control (unincub.) | 222.0 ± 4.5 | 154.5 ± 3.6 | 109.1 ± 2.7 |
|            | 30-min incub. | 63.6 ± 2.7 | 36.3 ± 1.8* | 36.3 ± 1.8* |
|            | 60-min incub. | 218.2 ± 4.5 | 90.9 ± 2.7 | 72.7 ± 2.7 |

Each value is the mean of 50 counts ± se. For each retinal piece, P values among the three zones were <0.001, except between values marked with asterisk where it was >0.05.

By the nature and arrangement of their organelles. In the inner, club-shaped portion, extending from the vitreous to the inner plexiform layer (Figs. 2, 9), the particles were numerous among the abundant microfilaments and did not appear to be attached or particularly related to the dilated smooth cisternae which are characteristic of the area (Figs. 2, 9). Beta particles were less numerous in the perinuclear area (Fig. 3), at the level of the inner nuclear layer, where most of the cell's rough endoplasmic reticulum and a large Golgi complex are situated, and were even scarcer in the outer cell portion (Fig. 4), from the outer plexiform layer outwards. Here, the particles were strewn among the abundant microtubules (Fig. 4) and the mitochondria which are clustered at the outer cell end.

In retinal neurons, beta particles were much less numerous than in Müller cells. These particles were seen in perikarya of ganglion cells and other nerve cells, as well as along dendrites in the neuropil. After lead staining, glycogen particles could be distinguished from ribosomes by their larger size and stronger contrast (Fig. 5). Mean diameters, as measured in ganglion cells, were 250 ± 1.2 (se) A and 215 ± 3.2 (se) A (P < 0.001 between both means), respectively, for beta particles and ribosomes. Glycogen particles were particularly conspicuous in the juxtanuclear region of ganglion cells, which contains the Golgi complexes and most lysosomes (Fig. 5). They were not observed in amylase-treated material (Fig. 6) or in untreated material stained with uranyl acetate. In these cases, the only particles observed had the diameter and the appearance of the ribosomes found in lead citrate-stained, nonamylase-treated material.

Effect of Incubation on the Retina

A pilot experiment revealed that synthesis of newly formed glycogen was higher after previous depletion of native glycogen. Hence, retinas were preincubated in medium lacking glucose for periods of 10, 20, and 30 min before the addition of the glycogen precursor. A 20-min period proved to be long enough to eliminate approximately all of the retinal glycogen visible in the light and electron microscopes. During preincubation, there were no appreciable morphological changes in Müller cells, but there was occasional shrinkage of nerve cell processes and enlargement of intercellular spaces, as well as a tendency for nuclei in rods and cones to become pyknotic. During further incubation in medium containing glucose, these
Figures 5–6  Electron micrographs of ganglion cell perikarya from unincubated retinas. Fig. 5 is from a specimen kept in buffer between glutaraldehyde and osmium tetroxide fixations and shows the juxanuclear cytoplasm region which contains the Golgi region (Go) and lysosomes (L). Beta particles (arrows) are larger and exhibit stronger lead affinity than ribosomes (R). Fig. 6 depicts a similar cell region but from a specimen treated with alpha-amylase between glutaraldehyde and osmium tetroxide fixations. All of the beta particles are absent from cytoplasm, while ribosomes were left. N, nucleus; Mi, mitochondria. Lead citrate. × 80,000.
changes increased slightly with time but were still moderate at 60 min, while in Müller cells the only change was an occasional lighter appearance of the background hyaloplasm.

**Synthesis of Glycogen**

In the course of incubation, beta particles of the same size, stainability, and distribution as in unincubated controls reappeared in the retina (Tables I, II). They were still scarce after 30 min but increased markedly at 1 hr of incubation, approaching the amounts observed in controls (Table II). The same results were obtained from light microscope observations: magenta material was rare at 30 min but was already abundant at 60 min, resembling the distribution of native glycogen. There were no differences in diameters of control and newly formed glycogen particles (Table I, Figs. 2-4, 9).

**Radioautography**

Silver grains appeared over the retinas incubated with glucose-3H. Their location in the light microscope was the same at both times of incubation, but the number of silver grains was much higher after 1 hr than after 30 min. The grains overlay the PA-Schiff-stained material and were more abundant and more uniformly distributed over the ganglion cell and nerve fiber layers (Fig. 7) where grain accumulations demarcated profiles that appeared to be those of Müller cells (Fig. 7). Hence, this region was preferred for grain counting in order to test the effectiveness of amylase digestion. Treatment of the sections with saliva removed much of the radioactivity, while alpha-amylase removed practically all of it (Fig. 8, Table III). The greater efficiency of purified alpha-amylase as compared with saliva confirmed previous results with incubated liver slices (6).

Electron microscope radioautographs showed many silver grains over the cytoplasm of Müller cells and a smaller number over the remaining retina including neuronal perikarya (Figs. 10, 11). In Müller cells, silver grains were evenly distributed over the cytoplasm (Figs. 9, 11), but their density per unit area of cytoplasm varied according to the cell portion; it was high in the inner portion

![Figures 7-8](downloaded_from_jcb.rupress.org)
TABLE III
Influence of Enzymatic Digestion on Radioactivity of Paraffin Sections Incubated with Glucose-3H

| Experiment | Incubation time | Control  | Alpha amylase |
|------------|-----------------|----------|---------------|
| I          | 30 min          | 69.1 ± 3.4 | 3.3 ± 0.4     |
|            | 60 min          |          |               |
| II         | 30 min          | 47.1 ± 3.2 | 7.0 ± 1.0     |
|            | 60 min          | 70.4 ± 4.4 | 9.0 ± 0.3     |
| III        | 30 min          | 71.6 ± 3.2 | 11.8 ± 0.9    |
|            | 60 min          | 85.0 ± 3.7 | 5.1 ± 0.7     |

Each value is the mean of 10 counts in one paraffin section after subtraction of background fog. Control sections were buffer treated.

DISCUSSION

Technical Considerations

The rabbit retina has been shown to be particularly adequate for in vitro studies (1). After 1-hr-long incubations in medium containing sodium and glucose, it continues to give normal electrophysiological responses and to present normal ultrastructure (1, 21). Prolonging the incubation in the same medium up to 2 hr provoked only occasional morphological changes, whereas withdrawing glucose from medium for 30 min was practically harmless (21). In our experiments, a high potassium medium was employed in order to enhance glycogen synthesis, and, under these conditions, a few moderate ultrastructural alterations were indeed recorded which mainly occurred during preincubation without glucose and consisted of shrinkage of nerve cell processes and enlargement of intercellular spaces. However, Müller cells showed no apparent alteration except an occasional lighter contrast of the background cytoplasm. Hence, the in vitro method was judged satisfactory for studying retinal glycogen synthesis at the ultrastructural level. The sites and the intensity of glycogen synthesis were evaluated, respectively, by the location and the density per unit cytoplasm area of silver grains.

Glycogen Synthesis in Müller Cells

Radioactive reactions showing glycogen synthesis were much more abundant over Müller cells than over the rest of the retina. No consistent associations between silver grains and any particular type of Müller cell organelle were, however, evident in electron microscope radioautographs. This fact and the even cytoplasmic distribution of the grains rather suggested glycogen synthesis to be a hyaloplasmic process. The in vitro method allowed total predepletion of the Müller cell native beta particles, so that those arising on addition of glucose were apparently entirely newly formed. Since the latter, like the native particles, were evenly distributed in cytoplasm, it was also easy to determine their densities per unit cytoplasm area, which, given their remarkable constant size, were taken to correspond with the local glycogen concentration. In this respect, Müller cell glycogen was unique if compared to glycogen of other cells, for example in liver (7) or muscle (8), in which the unequal distribution or the wide variations of particle size render particle counting per unit area meaningless.

Our principal finding, however, was the peculiar spatial distribution of the intensity of glycogen synthesis along the cell, which was considerably higher in the inner cell portion bordering the vitreous. This inner cell portion is situated at the level of the inner retinal layers in which it is generally assumed that anaerobic glycolysis prevails as an energy source (16, 19). It is thus suggested that local glycogen serves to yield glucose to the glycolytic pathway occurring in the inner retinal layers situated far away from the choroidal blood vessels which are the nearest source of oxygen and probably of glucose.

1 Although the vitreous contains some glucose, the concentration of it is much lower than in plasma (17) so that the role of the vitreous in supplying glucose to the retina must be negligible when compared to the role of choroidal vessels.
**Figure 9** Electron microscope radioautograph of the inner portion of the Müller cell. 18 silver grains are dispersed over the cytoplasm of two Müller cells (M₁, M₂). The grains are not particularly associated with the smooth cisternae (*) characteristic of this portion. Numerous beta particles are strewn over the cytoplasm. At left are the vitreous (V) and the inner limiting membrane. 1-hr incubation. Lead citrate. × 35,000.

**Figure 10** Electron microscope radioautograph of a ganglion cell (Ga.c.) perikaryon showing 11 silver grains in the Golgi (Go) region. L, lysosome; M, Müller cell. Lead citrate. × 20,000.
Figure 11  Electron microscope radioautograph showing the middle portion (M) of the Müller cell with the nucleus (N₂), and a bipolar nerve cell (bip.c.) with its nucleus (N₁). While 26 silver grains overlie the Müller cell cytoplasm, only two grains (arrow) appear over the nerve cell cytoplasm. Lead citrate. X 17,500.
This interpretation is strengthened by the predominant retinal location of glucose-6-phosphatase activity to the same cell portion (2, 15). Curiously, the latter is characterized by the existence of networks of smooth cisternae, which we were able to demonstrate as the real sites of glucose-6-phosphatase activity (unpublished results). Since no structures other than microfilaments, these smooth cisternae, and beta particles occupy the cytoplasm of this cell portion, it is tempting to assume that the inner end of the Müller cell is specifically concerned with storage and breakdown of carbohydrate. However, we do not know whether glucose liberated from glycogen diffuses to other cells or remains to be catabolized in it. Several histochemical studies (3, 13) have shown the Müller cell to be the principal retinal site of the lactic dehydrogenase which, by microchemical techniques, has been shown to prevail in the inner retinal layers (16, 19). Hence, it seems probable that the activities of anaerobic glycolysis occurring in the inner retina are more exactly located within Müller cell and that glucose derived from local glycogen is possibly broken down in this cell. In this case, the ATP molecules thereby produced might be transferred to, and utilized by, the remaining retinal cells.

The middle and outer cell portions are quite different from the inner portion in their ultrastructure, so that a definite segregation of organelles may be said to exist for each Müller cell portion. The middle portion is the only one provided with ergastoplasm, whereas the outer zone contains the only mitochondria of the cell, which, given the proximity of blood vessels, leads one to assume that some respiratory activity takes place in it. However, these cell zones also synthesize glycogen, and when the mean densities of silver grains per unit area of cytoplasm were plotted in logarithmic scale among the three zones, a straight outwards descending curve became manifest (Fig. 12). The synthesis thus declines in geometric proportion from the inner to the outer end of the Müller cell. In other words, one might say that glycogen synthesis and concentration rise inwardly with the distance from the irrigated choroid, which reinforces opinions that relate the abundance of glycogen to the lack of irrigation (14). Such a uniform geometric gradient is probably a reflection of a similar gradient in the concentration and activity of glycogen-synthesizing enzymes along the cell. However, although glycogen synthetase has been demonstrated in the retina biochemically, its cellular location has not been identified (10).

### Table IV
Comparison of Silver Grain Mean Densities per 100 µ² ± SS among the Three Portions of Müller Cell Cytoplasm and Ganglion Cell Cytoplasm

|                   | Experiment I | Experiment II | Experiment III |
|-------------------|--------------|--------------|---------------|
|                   | 30 min       | 60 min       | 30 min        | 60 min        | 30 min       | 60 min       |
| Müller cell       |              |              |               |               |              |              |
| outer portion     | 26.7 ± 3.77  | 44.2 ± 7.96  | 16.3 ± 4.03   | 31.7 ± 13.72  | 8.3 ± 2.15   | 15.1 ± 2.76  |
| P (Outer vs.     | >0.05        | >0.05        | >0.05         | >0.05         | >0.05        | >0.05        |
| middle)           |              |              |               |               |              |              |
| Middle portion    | 40.5 ± 5.55  | 57.0 ± 8.19  | 18.0 ± 3.26   | 42.9 ± 4.57   | 16.3 ± 5.29  | 12.1 ± 2.16  |
| P (Middle vs.     | <0.05        | <0.01        | <0.01         | <0.001        | <0.001       | <0.001       |
| inner)            |              |              |               |               |              |              |
| Inner portion     | 58.8 ± 5.15  | 89.7 ± 6.87  | 28.9 ± 2.24   | 103.8 ± 9.6   | 40.9 ± 9.11  | 63.1 ± 6.14  |
| P (Inner vs.      | —            | <0.001       | —             | <0.001        | <0.001       | <0.001       |
| G. cell)          |              |              |               |               |              |              |
| Ganglion cell     | 8.3 ± 0.81   | 13.4 ± 4.42  | 6.4 ± 1.28    | 16.3 ± 3.33   |              |              |
Glycogen Synthesis in Retinal Neurons

The cellular location of PA-Schiff-stained glycogenic material present in the retina outside of the main Müller cell profiles is difficult to determine with the light microscope, owing to the enormous intricacy of different cell processes. An electron microscope examination is needed, which allows demonstration of amylase-labile beta particles in retinal neurons. These particles are morphologically distinct from ribosomes by their larger size and stronger lead affinity. Glycogen particles are too few and too irregularly distributed to permit a quantitation of their amount, but it was possible by EM radioautography to study the rate of glycogen synthesis taking place in neurons, the number of silver grains over ganglion cell cytoplasm being usually large enough at 1 hr of incubation to allow determination of densities per unit area. Glycogen synthesis in the retina is, thus, no exclusive property of glial cells, which is interesting when compared with what occurs in brain. Here, also, glial cells have been thought to be the tissue carbohydrate reservoirs (22), but radioautographic studies performed with glucose-2H in the leech central nervous tissue (22) revealed glycogen synthesis to take place in both glial and nerve cells. However, while brain neurons convert glucose into glycogen more actively than glial cells (22), the reverse happens in the retina. Müller cells are, in fact, so preponderant in their capacity for glycogen storage that they may be taken as a separate type of glial cell which, besides having a supporting function, appears to be specialized in carbohydrate storage and delivery. Our findings indicate that such a property should be more particularly ascribed to the inner portion of the cell, and this may be of interest in clarifying the mechanisms of carbohydrate metabolism occurring in the inner retina. The mode in which that carbohydrate reserve is utilized by the tissue is a point to be pursued in future investigations.

Addendum: After acceptance of this paper we became aware of an abstract by Mauschinsky et al. (J. Histochem. Cytochem. 13: 707), in which a marked inward rise in the concentrations of glycogen, glucose-6-P, lactate, and UDPG across the rabbit retinal layers is reported. These microchemical findings are in agreement with our radioautographic data, and strengthen our conclusions.

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