

# Honeybee retinal glial cells transform glucose and supply the neurons with metabolic substrate

(retinal slices/2-deoxyglucose/glycolysis/glycogen/autoradiography)

M. TSACOPOULOS\*<sup>†</sup>, V. EVÉQUOZ-MERCIER\*, P. PERROTTET\*, AND E. BUCHNER<sup>‡</sup>

\*Experimental Ophthalmology Laboratory, University of Geneva School of Medicine, 22, rue Alcide-Jentzer, 1211 Geneva 4, Switzerland; and <sup>†</sup>Institut für Genetik und Microbiologie der Universität Würzburg, Röntgenring 11, 87 Würzburg, Federal Republic of Germany

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**ABSTRACT** The retina of the honeybee drone is a nervous tissue in which glial cells and photoreceptor cells (sensory neurons) constitute two distinct metabolic compartments. Retinal slices incubated with 2-deoxy[<sup>3</sup>H]glucose convert this glucose analogue to 2-deoxy[<sup>3</sup>H]glucose 6-phosphate, but this conversion is made only in the glial cells. Hence, glycolysis occurs only in glial cells. In contrast, the neurons consume O<sub>2</sub> and this consumption is sustained by the hydrolysis of glycogen, which is contained in large amounts in the glia. During photostimulation the increased oxidative metabolism of the neurons is sustained by a higher supply of carbohydrates from the glia. This clear case of metabolic interaction between neurons and glial cells supports Golgi's original hypothesis, proposed nearly 100 years ago, about the nutritive function of glial cells in the nervous system.

The hypothesis that one of the functions of glia in the nervous system is to transfer nutrients from the capillary blood to neurons was proposed by Golgi nearly a century ago (1, 2). This hypothesis seemed reasonable based upon histological evidence, but direct experimental support has so far been either meager or even negative (3). A question, often appearing in textbooks but as yet not answered, that bears upon the truth of this hypothesis is whether glycogenolysis in the glia serves to nourish the neurons. The question arises because histological observations show that in many nervous systems glial cells contain much more glycogen than neurons do (4-6). In the mammalian brain the glycogen is labile and it can be almost depleted by, for example, 1 min of ischemia (7) or hypoxia (8). Hence, it is known that carbohydrate stored as glycogen can be mobilized, but it is not known whether it is transferred from glia to neurons rather than being consumed by the glia themselves (9).

We have examined the nutritive role of glial cells in a comparatively simple nervous tissue, the retina of the honeybee (*Apis mellifera*) drone. In this preparation the separation of metabolic functions between the glial cells and photoreceptor cells (sensory neurons) is exceptionally complete. The photoreceptors contain large numbers of mitochondria and very little glycogen. In contrast, the glial cells contain very few mitochondria, but large quantities of glycogen  $\beta$  particles (10-12). Photoreceptor energy metabolism is obligatorily aerobic, since anoxia and the mitochondrial inhibitor amobarbital rapidly abolish light-induced electrical activity in the drone retina (11, 13). Because of these properties it is interesting to compare the requirement of photoreceptors for carbohydrate substrate with the changes in carbohydrate content of the tissue. A superfused retinal slice consumes about 18  $\mu$ l of O<sub>2</sub> per ml of tissue per min in the dark for up to 6 hr, and light stimulation of the photoreceptors almost quadruples this consumption in 2-3 sec and

for several hours even without exogenous substrate in the superfusate (12, 14, 15). The freshly dissected retina contains 56  $\pm$  11 mg of glycogen per ml of tissue (mean  $\pm$  SD) or 56/0.57 = 98.2 mg per ml of glia, where 0.57 is the volume fraction of the glial cells (16), but less than 10  $\mu$ M free glucose (1.8  $\mu$ g per ml of tissue) (12). We have calculated, by taking a respiratory quotient of 1, that to sustain respiration at least 31 mmol of hexose substrate per liter of tissue per hr must be transferred from the glia to the photoreceptors. Recent experimental evidence suggested that this carbohydrate is neither glucose [even though glucose is found at high concentration (72 mM) in the hemolymph] nor pyruvate, lactate, or trehalose (12). Direct evidence presented in this report shows that glucose obligatorily enters the glia, where it is transformed for transfer to the neurons. Also, photostimulation of the photoreceptor cells increases significantly the carbohydrate metabolism in the glia to sustain respiration in the photoreceptors.

## MATERIALS AND METHODS

**Preparation of Slices and Uptake of Labeled Hexoses.** Retinal slices ( $\approx$ 250  $\mu$ m thick) were prepared by making two parallel cuts with a vibrating razor blade, parallel to the ommatidia in the dorsal region of the retina, as described elsewhere (12). When such a slice is exposed to Ringer solution oxygenated with 100% O<sub>2</sub>, the retina is well oxygenated throughout its full thickness (14). The Ringer solution normally contained 270 mM NaCl, 10 mM KCl, 10 mM MgCl<sub>2</sub>, 1.6 mM CaCl<sub>2</sub> and 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.4. Each slice was labeled by incubation in Ringer solution containing 2-deoxy-D-[5,6-<sup>3</sup>H]glucose ([<sup>3</sup>H]dGlc; New England Nuclear; 66.2 Ci/mmol; 1 Ci = 37 GBq) at 200  $\mu$ Ci/ml (3.02  $\mu$ M) or D-[5-<sup>3</sup>H]glucose (New England Nuclear; 15.7 Ci/mmol) at 400  $\mu$ Ci/ml (26  $\mu$ M). During incubation the solution was stirred by a jet of moist oxygen, ensuring good oxygenation of the retina as checked with an O<sub>2</sub>-sensitive microelectrode. After the incubation time (45-60 min), the slice was rinsed for 20 sec in cold Ringer solution, frozen, and lyophilized. In some experiments the two retinas were separated; one was frozen immediately and the other retina was placed in a chamber and superfused with fully oxygenated Ringer solution for 60 min in the dark or while stimulated with light flashes. The viability of the preparation was checked by measuring the decrease in the partial pressure of O<sub>2</sub> (P<sub>O<sub>2</sub></sub>) induced by light stimulation. The retinas were microdissected from the brain (see figure 3 in ref. 12), weighed ( $\approx$ 120  $\mu$ g per retina), and homogenized by sonication in 600  $\mu$ l of 1 M HClO<sub>4</sub>. The total radioactivity was measured in aliquots of homogenate by liquid scintillation counting. The label incorporated into glycogen was measured following a specific extraction (17).

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Abbreviation: dGlc, 2-deoxy-D-glucose.

<sup>†</sup>To whom reprint requests should be addressed.

**Identification of Labeled Compounds.** The retinal homogenates were deproteinated by addition of  $\text{Ba}(\text{OH})_2$  and  $\text{ZnSO}_4$  and analyzed by high-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC). Isocratic HPLC was performed on a Bio-Rad Aminex HPX-87H column at 22°C with a Beckman 110A pump at a flow rate of 0.3 ml/min. Eluted radioactivity was measured in samples collected every 30 sec (Helirac 2212, LKB). Standard solutions (New England Nuclear products) were used for the identification of the peaks. Glucose and dGlc are well separated from their phosphorylated products by this method. The phosphorylated products were separated and identified by TLC as follows. A 100- $\mu\text{l}$  sample was injected into the HPLC column and the fractions of interest (Fig. 3A) were collected and pooled together, frozen, lyophilized, and dissolved again in 25  $\mu\text{l}$  of water. Spots of 10  $\mu\text{l}$  were made on TLC plates (plastic sheet cellulose, Merck 5577). The solvent system was ethyl acetate/acetic acid/pyridine/water (5:1.7:2.5:2.5, vol/vol). The separated species on the TLC plates were detected with a Berthold scanner (LD 2723).

**[ $^3\text{H}$ ]dGlc Autoradiography.** The method has been described in detail (18). The key to the high-resolution light microscope autoradiographic method for the localization of dGlc in sectioned tissue is the prevention of any diffusion of the water-soluble dGlc and dGlc 6-*P*. This was achieved by a procedure (18) that ensures that after freezing, the tissue is never exposed to aqueous solutions. The method of slice preparation and procedure for incubation with [ $^3\text{H}$ ]dGlc are described above.

**Stimulation.** All the photoreceptors in the superfused slices can be stimulated simultaneously and homogeneously by

light incident through a cut surface (19). Light stimulation was by means of a collimated light beam from a 150-W xenon lamp (model XBO 150, Osram, Munich). The beam was passed through neutral density and heat filters and an electronic shutter and was focused on the surface of the retinal slices with a microscope objective. With light stimulation oxygen consumption ( $\text{QO}_2$ ) increases (15), and with repetitive flashes (0.5 Hz) attenuated by  $-2.4$  logarithmic units ( $\approx 10^{14}$  photons per  $\text{cm}^2$  per sec), oxygenation from one surface is sufficient to a depth of 250  $\mu\text{m}$  (15). Thus only a negligible anoxic core was formed during photostimulation of these slices.

## RESULTS

**Uptake of dGlc by Retinal Slices.** We first asked this question: does glucose obligatorily enter the glia, where it is transformed for transfer to the neurons? We examined this question by incubating retinal slices in oxygenated Ringer solution containing [ $^3\text{H}$ ]dGlc. This molecule is transported through the cell membranes of the drone retina (12), as well as through other neuronal membranes (20, 21), presumably by the same mechanism as its structural analogue D-glucose. Phosphorylation of glucose to glucose 6-*P* and of dGlc to dGlc 6-*P* is catalyzed by the same enzyme, hexokinase, but dGlc 6-*P* is not metabolized further in the glycolytic pathway (22). After 60 min of incubation the retinal slices had accumulated  $2.37 \pm 0.05 \times 10^6$  cpm (mean  $\pm$  SD) of labeled material per mg of dry weight; of this, only 0.3% ( $6.2 \pm 2.3 \times 10^3$  cpm per mg of dry weight) was incorporated into glycogen. The magnitude of this incorporated portion is similar to that

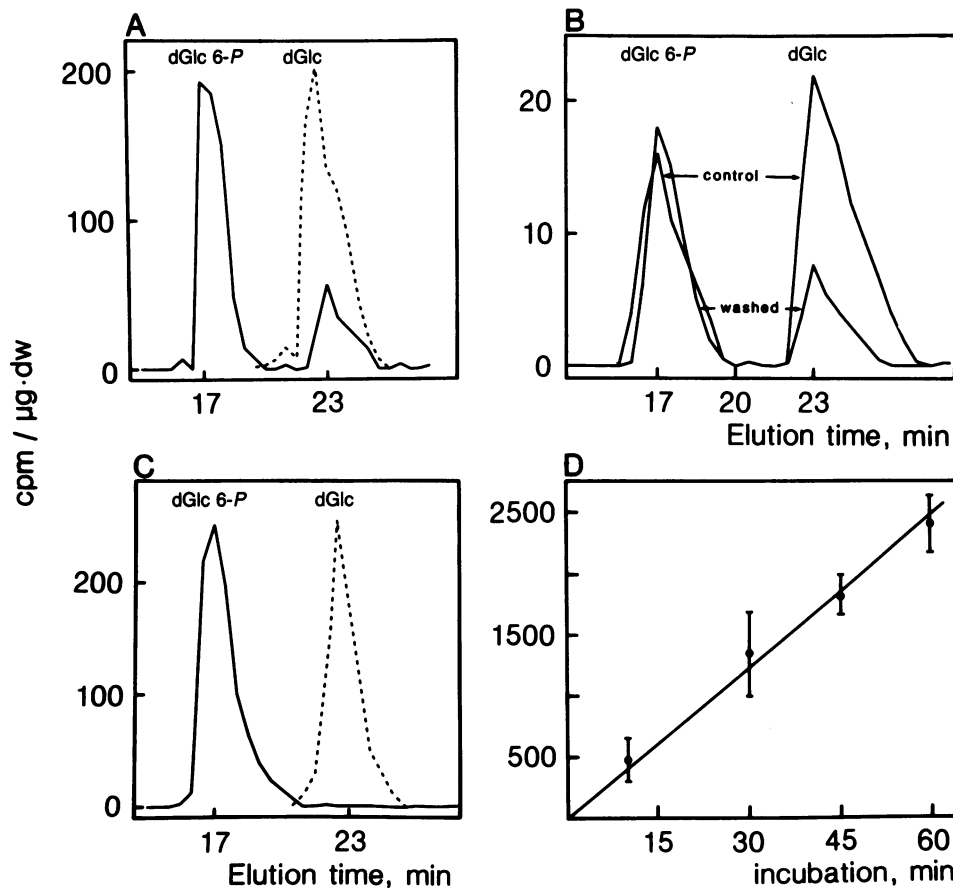


FIG. 1. (A–C) Chromatograms of homogenates of retinal slices incubated for 60 min with 3.0  $\mu\text{M}$  [ $^3\text{H}$ ]dGlc (A and C) or with 3.0  $\mu\text{M}$  [ $^3\text{H}$ ]dGlc plus 30 mM D-glucose (B). The dotted lines indicate the position of a [ $^3\text{H}$ ]dGlc standard. In A and in the experiment labeled “control” of B, the slices were frozen immediately after the incubation. In C and in the experiment labeled “washed” in B, the slices were washed after incubation. (D) Accumulation of [ $^3\text{H}$ ]dGlc 6-*P* as a function of time of incubation in 3.0  $\mu\text{M}$  [ $^3\text{H}$ ]dGlc followed by 30 min of washout. Each point is the average value from 7 slices, and bars are  $\pm$ SEM. Radioactivity is expressed as cpm per  $\mu\text{g}$  of dry weight (dw).

reported from experiments with the ganglia of snails (23). We performed HPLC of retinal homogenates to determine the molecular identity of the major portion of the radioactivity. The chromatogram of Fig. 1A shows two prominent radioactive peaks: one corresponds to dGlc 6-*P* (elution time, 17 min) and the other to dGlc (23 min). The ratio of the two peaks was 3:1 and similar to previous results with mammalian synaptosomes (24). The demonstration that the compound at 23 min was dGlc was provided as follows: the fractions corresponding to the peak were collected, frozen, and lyophilized; the residue was dissolved in water and mixed with a solution containing ATP and hexokinase for 15 min and analyzed again by HPLC. This time the peak appeared at an elution time of 17 min, corresponding to dGlc 6-*P*. When the retina was incubated with Ringer solution containing [<sup>3</sup>H]-dGlc (3 μM) plus 30 mM unlabeled D-glucose, the ratio of <sup>3</sup>H in dGlc 6-*P* to that in dGlc changed to 3:2.5 (average from 4 slices). A typical example (Fig. 1B) shows that less [<sup>3</sup>H]dGlc and [<sup>3</sup>H]dGlc 6-*P* accumulated in the presence of glucose than in its absence (compare Fig. 1A with Fig. 1C). During the wash with glucose-free Ringer solution, there was a small increase in [<sup>3</sup>H]dGlc 6-*P*. Probably the accumulation of glucose 6-*P* in the cells during incubation with 30 mM glucose inhibited the activity of hexokinase and, consequently, the transformation of dGlc to dGlc 6-*P* (25). Fig. 1C shows that when the slice was incubated for 60 min with [<sup>3</sup>H]dGlc and

then washed for 30 min in oxygenated Ringer solution, only [<sup>3</sup>H]dGlc 6-*P* was present in the chromatogram. Identical results were obtained from 10 different slices. The size of the [<sup>3</sup>H]dGlc 6-*P* peak decreased very little during 1 hr of wash-out (data not shown), indicating negligible activity of the enzyme glucose-6-phosphatase (21, 26). By using this experimental protocol, incubation followed by washing, we found that the accumulation of [<sup>3</sup>H]dGlc 6-*P* as a function of time of incubation was approximately linear up to at least 60 min (Fig. 1D). We can therefore calculate the amount of [<sup>3</sup>H]dGlc 6-*P* accumulated in the retinal cells during incubation. One curie is equivalent to  $2.22 \times 10^{12}$  dpm. The specific radioactivity of [<sup>3</sup>H]dGlc used was 66.2 Ci/mmol and the scintillation counter gave an efficiency of 40% (1 cpm = 2.5 dpm). Thus, 1 Ci =  $8.88 \times 10^{11}$  cpm. After 30 min of incubation the retinal slice preparation accumulated  $1.1 \times 10^6$  cpm/mg of dry weight (1 mg of dry weight = 3.3 mg of wet weight  $\approx$  3.3 μl of tissue). Thus, the concentration of [<sup>3</sup>H]dGlc 6-*P* in the retinal slice was  $[(1.1 \times 10^6 \text{ cpm/mg of dry weight}) \times (0.3 \text{ mg of dry weight}/\mu\text{l of tissue})] \div [8.88 \times 10^{11} \text{ cpm/Ci} \times 66.2 \text{ Ci/mmol}] = 5.6 \mu\text{M}$ .

The formation of [<sup>3</sup>H]dGlc 6-*P* was inhibited by addition of unlabeled dGlc to the incubation medium. In this experiment the slices were incubated in the presence of various concentrations of total dGlc (unlabeled plus [<sup>3</sup>H]dGlc): 0.128, 0.628, 0.940, 1.253, and 1.878 mM. After 30 min of incubation, the

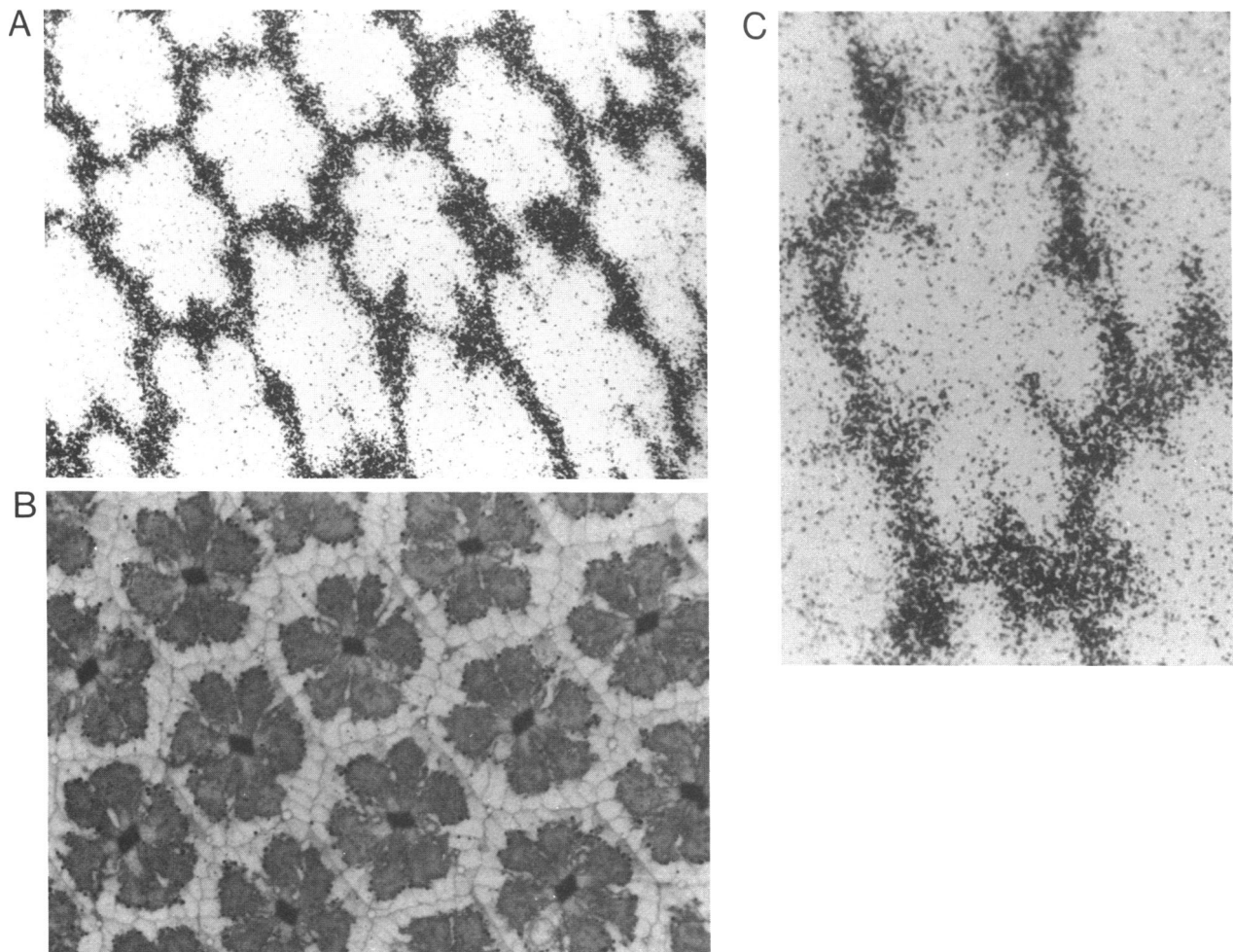


FIG. 2. (A) Autoradiograph of a section from a slice of drone retina that had been incubated with [<sup>3</sup>H]dGlc for 60 min and then washed for 30 min. ( $\times 655$ .) (B) Light micrograph of a section almost perpendicular to the axes of the ommatidia. The rosettes are the retinulae, clusters of six photoreceptor cells. The dark lozenge at the center of each retinula is the rhabdom. It consists of microvilli contributed by each of the photoreceptors and contains the photopigment. The pale cells between the retinulae are glial cells. Section was stained with Toluidine blue (10). ( $\times 640$ .) (C) Autoradiograph of a section from a slice (different than A) enlarged. The rosette is wrapped by glial cells labeled with [<sup>3</sup>H]dGlc 6-*P*. ( $\times 1065$ .)

corresponding intracellular concentrations of [ $^3\text{H}$ ]dGlc 6-*P* were 0.246, 0.914, 1.086, 1.310, and 1.451 mM. A double reciprocal plot gave a straight line with a corresponding  $K_m$  of 1.65 mM, not very different from that reported for vertebrates (24).

Hence, the uptake and phosphorylation of glucose in drone retina appear to be not very different from these processes in mammalian brain (21, 24).

**autoradiographic Localization of [ $^3\text{H}$ ]dGlc 6-*P* in the Drone Retina.** To see which cells took up radiolabeled material, we made autoradiographs of the drone retinas that had been incubated with [ $^3\text{H}$ ]dGlc for 60 min and then washed for 30 min. Autoradiography showed radioactivity only in the glial cells (Fig. 2A). The stained histological section of the drone retina shown in Fig. 2B helps in identifying the two cell types, glial cells and photoreceptors, in the autoradiograph; several retinulae are recognizable (Fig. 2A and C). Control autoradiographs of slices frozen after 60 min of incubation with 3  $\mu\text{M}$  *O*<sup>3</sup>-methyl-D-[ $^3\text{H}$ ]glucose showed weak but uniform labeling of both cell types (see also ref. 18). *O*<sup>3</sup>-Methyl-D-glucose is a nonmetabolizable sugar that is transported into the retinal cells (12) but not phosphorylated by hexokinase (22). *O*<sup>3</sup>-Methyl-D-[ $^3\text{H}$ ]glucose was washed out of retinal slices with a half-time of about 15 min (12). Autoradiographs made after 30 min of washing showed no labeling above the background level. This demonstrates that accumulation of label is specific for dGlc and depends on the reaction with hexokinase and the accumulation of the phosphorylated product in the cell.

The biochemical data presented above showed that after 60 min of incubation in [ $^3\text{H}$ ]dGlc followed by 30 min of washout, only labeled dGlc 6-*P* and glycogen were present in the retina. The following calculation demonstrates that the labeling in the autoradiograph is due essentially to [ $^3\text{H}$ ]dGlc 6-*P*. About 100 disintegrations are needed to make about 10 grains in the autoradiographs (27). The retinal sections used for autoradiography in this study were 4  $\mu\text{m}$  thick, but  $^3\text{H}$   $\beta$ -radiation penetrates only  $\approx 1 \mu\text{m}$  of Epon (the fixative used here). The diameter of an ommatidium is about 30  $\mu\text{m}$  (10), so its volume in a 1- $\mu\text{m}$  section is 0.9 pl. The radioactivity in dGlc 6-*P* and glycogen after 60 min of incubation followed by 30 min of washout was, respectively,  $2.375 \times 10^6$  and 6943 cpm/mg of dry weight. The exposure time of the photographic films was 12 days. Thus [ $^3\text{H}$ ]dGlc 6-*P* would make about 2800 grains per ommatidium cross-section in the autoradiography and labeled glycogen about 8 grains per ommatidium. Taking the diameter of a grain as  $\approx 0.5 \mu\text{m}$ , if one placed the 2800 grains only in the glial cells of one ommatidium, one would obtain the heavy labeling shown in the autoradiograph of Fig. 2C. We conclude that the labeling in the autoradiograph is due essentially to [ $^3\text{H}$ ]dGlc 6-*P* and, thus, that only glial cells are able to convert glucose to glucose 6-*P*.

**Effect of Light Stimulation on Carbohydrate Metabolism.** Since glycolysis occurs only in the glial cells and mitochondrial respiration only in the photoreceptors, the following question arose: what happens to glucose metabolism in the glia during stimulation? This question is interesting because in the drone retina only photoreceptors are directly excitable by light, since they contain the visual pigment rhodopsin (28). In these experiments, retinal slices were loaded with [ $^3\text{H}$ ]glucose, and the accumulation of radioactivity in the retina and the incorporation of [ $^3\text{H}$ ]glucose into glycogen were measured as a function of time of incubation. There was no sign of saturation for incubation times up to 60 min. HPLC showed that after 60 min of incubation the ratio of phosphorylated [ $^3\text{H}$ ]glucose to nonphosphorylated was about 3:1 (Fig. 3A). In the control retina the average radioactivity after 60 min of incubation was  $808 \pm 359 \times 10^3$  cpm/mg of dry weight (mean  $\pm$  SD,  $n = 53$ ). Because of this high variability, we chose to compare one retina to the other in the same slice

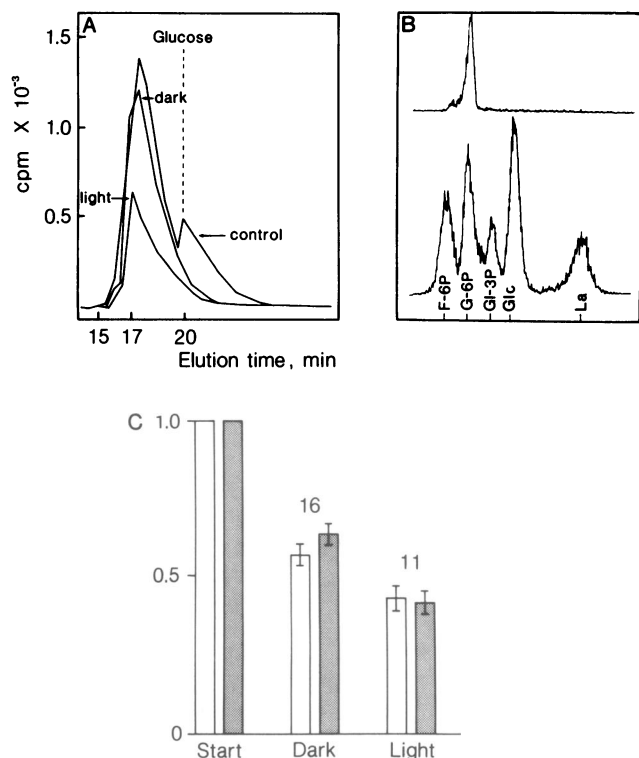


FIG. 3. (A) HPLC chromatograms of homogenates of retinal slices incubated for 60 min with 26  $\mu\text{M}$  [ $^3\text{H}$ ]glucose. In the control the slice was frozen immediately after the incubation, whereas in the two other experiments the slice was superfused for up to 60 min either in the dark or while stimulated with light flashes, one every 2 sec. Broken line indicates the position of glucose. (B) TLC. Lower trace, standards representing the major glycolytic products are adequately separated (F-6P, fructose 1,6-*P*<sub>2</sub>; G-6P, glucose 6-*P*; Gl-3P, glycerol 3-*P*; Glc, glucose; La, lactate). Upper trace, chromatogram of the "dark" peak in A. Only one phosphorylated glycolytic intermediate is present. (C) Effects of light on glucose metabolism. Remaining total radioactivity (open columns) and the radioactivity in glycogen (stippled columns) were measured after 60 min of superfusion under dark or light conditions as described in A. The values are expressed as a ratio between the experimental retina, which was either washed in the dark or under light stimulation, and the control retina (from the same slice), which was frozen immediately after incubation in the radioactive solution. The number of slices is indicated above each column. Error bars are SEM. The difference between dark and light is statistically significant ( $P = 0.012$  for the total radioactivity and  $P < 0.001$  for the radioactivity incorporated into glycogen).

(see *Materials and Methods*). After 60 min of incubation, one retina was rinsed and frozen as a control while the other was placed in a chamber and superfused with oxygenated glucose-free Ringer solution under the chosen experimental conditions. In all retinas, the major labeled carbohydrate was a phosphorylated product of [ $^3\text{H}$ ]glucose, probably [ $^3\text{H}$ ]glucose 6-*P* (Fig. 3B). Other labeled products of glycolysis were not detected, possibly because the label, which was at position 5, was lost by metabolism of glucose 6-*P* to fructose 6-*P*, fructose 1,6-*P*<sub>2</sub> to glyceraldehyde 3-*P*, or 2-phosphoglycerate to phosphoenolpyruvate (29). In retinas frozen immediately after 60 min of incubation,  $\approx 1\%$  ( $0.99 \pm 0.06\%$ , mean  $\pm$  SEM,  $n = 31$ ) of the label was incorporated into glycogen. During the wash both total radioactivity, mainly in [ $^3\text{H}$ ]glucose 6-*P* (Fig. 3B), and radioactivity incorporated into glycogen decreased (Fig. 3C), probably due to the metabolism of glucose 6-*P* to other products. This parallel decrease indicates that there is a steady-state equilibrium between glucose 6-*P* and the glycosyl units of glycogen. Light stimulation of the photoreceptors increased the rate of breakdown

of [<sup>3</sup>H]glucose 6-*P* and of [<sup>3</sup>H]glycosyl incorporated into intraglial glycogen (Fig. 3C).

### DISCUSSION

The results show that in a metabolically compartmented nervous system such as the isolated drone retina, glial cells, but not sensory neurons, take glucose and convert it to glucose 6-*P*. In insects the retina is sealed off from the blood (hemolymph) by an electrical and diffusional barrier, the basal membrane (30). Autoradiographs made after [<sup>3</sup>H]dGlc injection into the blood of intact flies (18) and drones (V.E.-M. and M.T., unpublished data) showed considerable amounts of radioactivity in the retina, but the photoreceptors were not labeled. Thus, it appears likely that, in living insects, carbohydrates are transported from the blood to the retinal glia either directly, at the sites of contact between the basal membrane and the glial cells (30), or via the retinal extracellular fluid compartment. The glial cells transform glucose through the glycolytic and glycogen pathways and some, as yet unidentified, product feeds the sensory neurons continuously. Previously, we showed (17) that stimulation of the photoreceptors in the living drone modifies the metabolism of the glycogen in the glia. In the present work, we examined further the nature of the biochemical modifications in the metabolism of glucose by taking advantage of the possibilities of the slice preparation. Photostimulation of slices superfused with substrate-free Ringer solution quadruples the O<sub>2</sub> consumption of the photoreceptors and increases in the glia the rate of breakdown of [<sup>3</sup>H]glucose 6-*P* and of incorporation of [<sup>3</sup>H]glycosyl into glycogen. After 2 hr of repetitive stimulation the concentration of total glycogen decreased significantly, by 14 ± 4 mg per ml of tissue (mean ± SEM). This amount is more than sufficient for the carbohydrate requirements calculated from the O<sub>2</sub> consumption. Consequently, those results demonstrate that the increased oxidative metabolism of the sensory neurons is sustained by a higher supply of carbohydrates from the glia. Even though the molecular mechanisms are not yet elucidated, the drone retina offers a clear case of metabolic interaction between neurons and glial cells, supporting about 100 years later Golgi's original hypothesis.

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