Localization of phosphorylase in the alloxan-diabetic rat retina

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Phosphorylase was demonstrated histochemically in the retina of normal and alloxan-diabetic rats. In the normal rat, phosphorylase was present in the inner segments of visual cells, nuclear layers, plexiform layers, ganglion cell layer, and the nerve fiber layer, and was predominant in the inner layers of the retina. The localization of phosphorylase in the alloxan-diabetic rat retina was generally similar to that in the normal rat retina, but the intensity was markedly increased, particularly in the inner layers of the retina. In general, the distribution of phosphorylase corresponded to that of glycogen; however, phosphorylase was present in the absence of detectable glycogen.

Phosphorylase is an enzyme which, in conjunction with the branching and debranching enzymes, catalyzes the reversible reaction: glycogen to glucose-1-phosphate. A method for the histochemical identification of phosphorylase was first described by Yin and Sun, who demonstrated the enzyme in plant tissues. Cobb demonstrated animal phosphorylase histochemically in sections of frozen dried cartilage. The phosphorylase method was extended by Takeuchi and Kuriaki, who used activators (insulin and adenosine-5-phosphate) and a primer (glycogen). Eränkö and Palkama described improved results by adding polyvinyl pyrrolidine and an excess of glucose-1-phosphate to the incubating medium developed by Takeuchi and Kuriaki.

A number of investigators have identified the enzyme in various tissues, but there are few recorded histochemical studies of the enzyme in the ocular tissues. The present investigation was undertaken to study distribution of phosphorylase and its relation to glycogen localization in the retina of alloxan-diabetic rats.

Method

Male albino Sprague-Dawley rats weighing between 312 and 355 grams received 20 mg. of alloxan per 100 gram of the body weight subcutaneously and were fed a standard Purina rat chow. Seven of the rats were sacrificed 7 months later for this investigation. An equal number of untreated animals were kept for the same period as controls.

The alloxan-diabetic rat urine was repeatedly positive for reducing substance. Blood glucose levels ranged from 460 to 680 mg. per 100 ml. in the fourth month after the alloxan injection. On the day of sacrifice the diabetic rats weighed between 156 and 180 grams and blood glucose levels were 518 to 690 mg. per 100 ml. Cataract was present in all. The control rats weighed 560 to 620 grams, the blood glucose levels were 72 to 98 mg. per 100 ml., and no cataracts were present.

The animals were anesthetized with ether and their eyes enucleated. The eyes were cut open at the equator and the vitreous was removed im-
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mediated. In half the eyes the remaining portion was fixed in 100 per cent ethanol. The entire procedure was carried out in daylight and at room temperature. After being dehydrated and embedded in paraffin, sections 8 μ in thickness were treated with periodic acid, and then stained with leukofuchsin.11 Counterstaining was done with Harris’ hematoxylin solution. Other sections were treated with salivary amylase for one hour to remove glycogen.

The posterior half of the fellow eye was immersed in a solution of isopentane, kept at -70° C. with a mixture of dry ice and acetone. The freshly frozen tissue was mounted on a chuck by being frozen in place with water. Sections 15 μ in thickness were cut with a cold microtome (cryostat) at a temperature of -20° C. and mounted on cover slips. The cover slips with mounted alloxan-diabetic and normal sections were placed in the incubating medium of Eräskö and Palkama at 37° C. The sections were removed from the incubating fluid after 3 hours and allowed to dry. They were then immersed in 0.32 M sucrose for 5 minutes, stained with Crum's iodine solution containing 0.32 M sucrose for 5 minutes and mounted with glycerin jelly. The site of phosphorylase activity indicating enzymatic synthesis of amylose-type polysaccharide appeared blue-black. The control sections were placed in a similar incubating solution which did not contain glucose-1-phosphate.

**Results**

In the normal rat no phosphorylase reaction was observed in the pigment epithelium or in the outer segments of visual cells. A few granules with the blue-black color indicating phosphorylase activity appeared in the inner segments of some visual cells. However, this region was usually free of the enzyme. The outer nuclear layer and external limiting membrane showed no reaction, but in some areas fine blue-black granules were arranged perpendicularly to the plane of the retinal surface among the visual cell nuclei. The outer plexiform layer was stained diffusely deep blue-black and showed the highest phosphorylase activity. In the inner nuclear layer, many fine, deep blue-black granules arranged dendritically among the nuclei were observed. The inner plexiform layer showed a slightly positive reaction, and, in the middle of the layer, a strongly staining zone which paralleled the surface of the retina was present. A moderate to strong reaction for phosphorylase was observed in the ganglion cell layer, but not in the ganglion cell nuclei. The fine blue-black granules were present in the cytoplasm of some ganglion cells. The nerve fiber layer showed a weak reaction for phosphorylase and in some areas was stained deep blue-black. In the region near the disc phosphorylase activity appeared on the fibers passing through the nerve fiber layer in vertical direction to the retinal surface. Some parts of the internal limiting membrane took a blue-black stain (Fig. 1, left).

The localization of phosphorylase in the retinas of the alloxan-diabetic rats was generally similar to that in the normal rats. However, the intensity of the reaction was markedly increased, primarily in the inner layers of the retina. The pigment epithelium and inner and outer segments of the visual cells exhibited no increase of phosphorylase. The outer limiting membrane showed a weak reaction, staining faint blue. The outer nuclear layer was also stained faintly, but not the nuclei. In many places in the outer nuclear layer blue-black granules appeared arranged vertically among the visual cell nuclei. The outer plexiform layer stained strongly and had a greater number of the granules than that of the normal rat retina. These granules were located diffusely throughout the layer. In the inner nuclear layer, the accumulation of the granules was evidently increased, and some nuclei in the layer were covered by the granules. The inner plexiform, ganglion cell, and nerve fiber layers were stained deeper than the same layers of the normal rat retinas. Thin fibers, stained blue-black and running perpendicularly to the retinal surface from inner to limiting membrane, were outlined through the retina in some sections. In the nerve fiber layer near the disc, phosphorylase activity was also intensified along the fibers in a vertical direction. Some parts of the internal limiting membrane had the blue-black granules (Fig. 1, right; Figs. 2 and
3). No phosphorylase was found in the retinal vessels in the diabetic or normal rat retinas.

A few glycogen granules in the inner segments of some visual cells were found. Little or no glycogen was observed in the outer nuclear, outer plexiform, inner nuclear layers, and in the inner plexiform layer. Slight amounts of glycogen was present in the ganglion cell layer. The nerve fiber layer had glycogen granules arranged perpendicularly to the nerve fiber bundles. Glycogen granules were sometimes seen on the internal limiting membrane (Fig. 4, left). In the diabetic retina, deposits of glycogen were increased in the inner plexiform, ganglion cell, and nerve fiber layers, and also in the internal limiting membrane. In the inner nuclear layer and outer plexiform layer, the glycogen reaction was slightly pronounced. Some of the glycogen granules were arranged along fibers extending through the inner plexiform layer in a vertical direction. The fibers were outlined through the nerve fiber, ganglion cell, and inner plexiform layers. No increase was observed in the outer layer of the retina (Fig. 4, right). No evidence of microaneurysms, capillary hemorrhage, sclerosis of the vein, and proliferative retinitis could be found in the diabetic rat retinas.

Figs. 1-4. Key to abbreviations. N, Nerve fiber layer; GC, ganglion cell layer; IP, inner plexiform layer; IN, inner nuclear layer; OP, outer plexiform layer; ON, outer nuclear layer; V, rods and cones; P, pigment epithelium; O, optic nerve.
Fig. 1. Left: Normal rat. Phosphorylase activity is indicated by the black areas in the plexiform, ganglion cell, and nerve fiber layers, as well as the inner nuclear layer. There is no activity in the rods and cones. Right: Alloxan-diabetic rat. Phosphorylase activity is pronounced in the inner retinal layers (×425; reduced ½.)
Fig. 2. Alloxan-diabetic rat near the papilla. Phosphorylase activity is observed in the nerve fiber layer in some areas. Intense phosphorylase activity is seen in the ganglion cell, plexiform and inner nuclear layers. (×195; reduced approximately ⅓.)

Fig. 3. Alloxan-diabetic rat. The phosphorylase activity is noted along fibers running perpendicular to the retinal surface. (×425; reduced approximately ⅓.)

Discussion

Nagaya found phosphorylase activity in chicks in those parts of the inner segments of cones in which glycogen was present, in some of the cone feet, in the inner plexiform layer, especially at its outer half along supporting fibers, in the nerve fiber layer, and at the internal limiting membrane. Phosphorylase in the frog retinas was distributed in the inner segments of cones, plexiform layers, nerve fiber layer, internal limiting membrane, and supporting fibers.
The outer plexiform layer had the greatest phosphorylase activity. Ohashi, studying rabbits, found a slightly positive phosphorylase reaction in inner and outer segments of the photoreceptor cells and a more marked reaction in the inner retinal segments. Viale and Apponi found intense phosphorylase activity in the plexiform layers of the human retina.

The distribution of phosphorylase in the retinas of the normal rats was generally similar to that previously described; the phosphorylase activity was predominant in the inner layers of the retina, despite the differences in glycogen distribution in the retinas of various species. The amount and localization of phosphorylase did not parallel that of glycogen in the previous studies. In the retinas of normal rats, the localization of phosphorylase did not parallel the distribution of glycogen, especially in the outer plexiform and inner nuclear layers, in which little or no glycogen was found but in which there was a strong phosphorylase reaction. In the diabetic rat retinas, the increase in phosphorylase generally paralleled the increase in glycogen. Arizawa found glycogen in all the retinal layers of alloxan-diabetic rabbits. Nakashima reported glycogen deposits in the pigment epithelium of the rabbit retina after subcutaneous and intravitreal injection of dextrose. Best reported deposits of glycogen in the pigment epithelium of the diabetic human retina. In the present investigation, neither glycogen nor phosphorylase was found in the pigment epithelium of the diabetic rat retinas.
Phosphorylase activity in Müller’s fiber was noted by Nagaya and Ohashi. Kuwabara and Cogan found that glycogen was synthesized and stored in Müller’s cell of the retina of various species, but little or none was present in the rat retina. However, glycogen increased, especially in Müller’s fibers after perforating wounds of the rat eye. In the present study, phosphorylase and glycogen deposits were found arranged perpendicularly to the plane of the retinal surface. However, it was not possible to determine whether the deposits were in Müller’s fibers or in the axons or dendrites of nerve cells.

Opinions concerning the role of the glycogen in retinal metabolism have been varied; e.g., Schmitz-Moormann considered glycogen in the retina an energy source for contraction of cones, while Shimizu and Maeda regarded the glycogen as the energy source for restoration of rhodopsin, phosphocreatine, and adenosine triphosphate. Conversely, with the exception of glycogen storage disease, which, in general, shows no hyperglycemia, and some types of enzyme deficiency (glucose 6-phosphate, branching enzyme, debranching enzyme and phosphorylase), abnormal accumulations of glycogen in the cells of the body are a reflection of hyperglycemia of which diabetes mellitus is the most important cause. The glycogen deposits represent an individual cell accumulation of glucose in response to hyperglycemia and are most important as anatomic indicators of deranged carbohydrate metabolism.

Although phosphorylase synthesizes glycogen in vitro, the enzyme is concerned in vivo only with glycogen degradation. The pathway of glycogen synthesis in vivo is from uridine diphosphate glucose through the uridine diphosphate glucose-glycogen transferase system. Nagaya found that glycogen appeared in the chick embryo retina on the eighteenth or twentieth day of development, and phosphorylase on the twenty-first day. The late appearance of phosphorylase indicates that the chick embryo retina can synthesize glycogen without phosphorylase.

In our study, the accumulation of glycogen in the diabetic rat retina was accompanied by increased phosphorylase activity. The increased amounts of phosphorylase in the diabetic rat retina may result from increased synthesis and degradation of the excess glycogen. The glycogen accumulation in the diabetic rat retina may be due to a greater increase in the rate of glycogen synthesis compared to the rate of glycogen degradation which may be less markedly increased. However, the uridine diphosphate glucose-glycogen transferase system, through which glycogen synthesis is performed in vivo, could not be demonstrated histochemically in the normal or diabetic rat retina.

Another possibility is that the increase of phosphorylase in the alloxan-diabetic rat retina may result from an increase of phosphorylase B, the inactive form of the enzyme. Possibly, as suggested by Bárany, native glycogen acts as a primer for the phosphorylase. Thus Nagaya found no phosphorylase activity in the retina treated with amylase prior to incubation in his medium, which contained no glycogen. However, phosphorylase activity was found in the retina in the absence of demonstrable glycogen in the current study. Further investigation is required to clarify this point.

The detection of phosphorylase activity may be a more sensitive indicator of carbohydrate metabolism than periodic acid-Schiff glycogen staining. The abnormal increase of phosphorylase activity and abnormal accumulation of glycogen in the diabetic rat retina are manifestations of disturbances of carbohydrate metabolism in the diabetic rat retina. Thus, it is interesting to recall the hypothesis that disturbances of metabolism in the retina pre-
cede diabetic retinopathy, which has been presented by Ashton,\textsuperscript{23} and de Roeth and Yen.\textsuperscript{24}

Addendum

Hutchinson and Kuwabara\textsuperscript{25} have recently reported that phosphorylase activity has been demonstrated histochemically in the inner segments of the retinas of various species, and that minimal uridine diphosphoglucose glycogen synthetase activity in the diabetic human retina and the calf retina has been demonstrated by histochemical methods, and the presence of the enzyme has been shown biochemically in the calf retina.

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REFERENCES


Discussion

Dr. B. Thomas Hutchinson, Boston, Mass. Drs. Kurimoto and Newell have nicely demonstrated the presence of phosphorylase activity in the retina of the normal and the alloxan-diabetic rat and have presented a lucid, concise discussion of the metabolism of retinal glycogen. Previously, Kuwabara and Cogan reported the distribution of retinal glycogen in a variety of animals and de-
scribed a species variation in native glycogen content which showed glycogen, as demonstrated histochemically, to be roughly inversely proportional to the vascularity of the retina.

Using essentially the same histochemical technique as employed in this presentation, Dr. Kuwabara and I have studied phosphorylase in the retinas of a variety of species; we, too, have found abundant phosphorylase activity to be distributed throughout the inner segments of the retina in all species, regardless of the relative native glycogen content as demonstrated histochemically. Thus the mouse, having no histochemically demonstrable glycogen, the rat, having glycogen only in the nerve fiber and ganglion cell layers, and the guinea pig, having glycogen throughout the inner portions of the retina, have identical distributions of phosphorylase.

We have been unable to demonstrate consistently the presence of UDPG glycogen synthetase in the retina by histochemical techniques but have been able, by enzymatic assay, to demonstrate this enzyme to be present in the calf, rat, and mouse retinas. It seems that synthetase, perhaps normally attached to particulate glycogen, is solubilized by freezing and is thus not histochemically evident when “cryostat” techniques are employed.

We share the authors’ view that the localization of phosphorylase activity might give a better indication of sites of significant glycogen turnover than is given by the histochemically demonstrable glycogen itself. Certainly the normal species variation of retinal glycogen offers to the histochmist and the biochemist an unusual opportunity, not found in other tissues, to further explore differences in this important compound.