

Quantitative Histochemistry of Retina

II. ENZYMES OF GLUCOSE METABOLISM*

OLIVER H. LOWRY, NIRA R. ROBERTS, DEMOY W. SCHULZ, JANE E. CLOW, AND
JAMES R. CLARK

*From the Department of Pharmacology and the Beaumont-May Institute of Neurology,
Washington University, St. Louis 10, Missouri*

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It was previously reported that lactic and malic dehydrogenases are distributed according to strikingly different patterns in the various layers of the retina (1). This suggested that the layers differ greatly in regard to glycolytic and oxidative capacity. In this paper will be reported the distribution of the following enzymes of glucose metabolism: hexokinase, phosphoglucoisomerase, phosphofructokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and phosphoglucomutase. This permits examination of most of the enzymes leading from glucose through glucose 6-phosphate toward each of three major pathways: glycolysis, direct oxidation, and glycogen formation.

The differences in distribution of certain of these enzymes are dramatic and may indicate how the retina meets some of its special problems. Comparison of the avascular retina of the rabbit with the partially vascularized retina of the monkey (*Macaca rhesus*) helps in the assessment of the effects of blood supply on metabolic patterns.

Some of the analytical methods are new and perhaps of interest *per se*.

EXPERIMENTAL PROCEDURE

The preparation of the histological material for analysis has been described (1, 2). The analyses were made on pure samples of each retinal layer. These were obtained by dissection from frozen-dried sections cut at 5 or 6 μ in a plane parallel to the retinal layers. The average sample weighed 0.1 μ g.¹

It was desirable to use a single set of sections for many determinations. The enzymes measured had been shown to be stable in dry sections stored for long periods at -20° . It was also known that exposure to room temperatures for the few hours required to dissect samples for a single set of analyses could be

tolerated by the enzymes being studied. However, it was not known if repeated exposure to room air would be tolerated. A trial with monkey retina sections (6 μ) gave the following results. Hexokinase, 6-P-gluconate dehydrogenase, and glucose-6-P dehydrogenase lost, respectively, 75, 84, and 100% activity after 11 days in air at $25-28^{\circ}$, and 45, 41, and 65% activity after 3 days in air at 25° . When, instead, samples were stored for 3 days at 25° under vacuum, hexokinase lost no activity and 6-P-gluconate dehydrogenase lost only 7% activity. Glucose-6-P dehydrogenase still lost considerable activity (45%) under vacuum at 25° .

Whether the loss in air was due to moisture, oxygen, or adventitious fumes was not determined. As a result of this experiment, it has become the practice to remove only a few samples at a time from the vacuum tube (2) for dissection and to keep the rest of the samples under vacuum until the dissection is finished, at which time all remaining material is returned to -20° (under vacuum).

The enzyme analyses were all performed with samples dissected on the day of analysis. In this type of microanalysis, the most hazardous step has been found to be the bringing together of dry sample and buffer-substrate reagent in the reaction tube (2-mm bore). Placing tubes with dry samples in an ice bath before addition of reagent, or storing samples in tubes at low temperature after dissection may be disastrous because of condensation of moisture (3). The procedure recommended, and used for much of this study, is to add complete reagent to each tube *at room temperature* and at once bring the sample into the reagent under direct supervision through a wide angled, low power microscope. For this purpose, a simple rack and pinion device is required (3). Immediately after receiving the section, the tube is placed in a rack in ice and held until the set is complete. Alternately, the order of addition was reversed, but the operation was still performed at room temperature. This simpler procedure has the disadvantage that supervision is very difficult. The samples, invisible to the naked eye after they are wet, may stick to the bottom, be carried high into the meniscus, or be carried out of the tube on the pipette tip. Repeatedly, tests have shown that after bringing sample and reagent together, physical mixing is unnecessary and undesirable. Diffusion and convection provide adequate mixing with the small fluid volumes concerned.

After incubation, the racks of samples, standards, and blanks were placed in ice to arrest enzyme action temporarily before proceeding with the analyses.

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¹ When the frozen sections were cut, a considerable improvement was made by mounting the entire eyeball in a holder of hard wood. The holder made on a lathe has a stem approximately 3 cm long and 1.2 cm in diameter with a flared end containing a socket-shaped depression like a shallow eye cup. The eyeball (previously frozen as rapidly as possible) is mounted in brain paste with the corneal pole in the socket. Freezing to the paste is accomplished by covering with powdered dry ice because cracking might result from too rapid chilling. The holder plus eyeball can be turned to present almost any portion of the fundus to the blade. The sclera is chipped away from the choroid with a scalpel over the region to be sectioned. After sectioning, the remainder of the eyeball, still in its cup, may be preserved at -80° for further sampling.

All the methods used are based finally on measurement of TPNH or DPN⁺ by fluorescence. TPNH was measured either by its native fluorescence or by the stronger fluorescence produced in strong alkali after conversion back to TPN⁺ with hydrogen peroxide (4). All readings were made in selected Pyrex tubes of 10- × 75-mm outer diameter in the Farrand model A fluorometer.

When the native fluorescence is to be read with TPNH concentrations below 5×10^{-7} M it is advantageous to measure the blank fluorescence of each tube containing the diluent to be used, before adding the aliquot containing TPNH. This blank reading need not average more than the fluorescence from 10^{-7} M TPNH, but it is likely to be somewhat variable and is contingent on the quality of tube cleaning, and the quality of the water used in preparing the diluent.

When the fluorescence inducible with strong alkali is to be measured, it is necessary to destroy excess TPN⁺ beforehand with minimal fluorescence formation. Originally, this was accomplished by heating in 0.03 N NaOH (4). An improvement permitting greater latitude in sample volumes and buffer strengths is to use a strong solution of K₂HPO₄, and Na₃PO₄ to give a final ratio of HPO₄²⁻:PO₄³⁻ between 1.5 and 3. Heating for 10 minutes at 60° in this buffer destroys TPN⁺ (or DPN⁺) with formation of fluorescence equal to less than 1% of that formed in 6 N NaOH.

Standards, in all cases, were prepared in the complete buffer-substrate reagents at the concentrations indicated, and these were incubated together with the tissue samples.

Two of the enzymes measured produce TPNH directly. Three others yield glucose-6-P from which TPNH was formed by the addition of TPN⁺ and glucose-6-P dehydrogenase. This enzyme was prepared from hog adrenals (5) and was nearly free of hexokinase and P-glucoisomerase.² The Michaelis constant, K_s , for glucose-6-P at pH 8 is about 0.02 mM, and that for TPN⁺ about 0.002 mM. In the case of phosphoglucomutase and isomerase, the auxiliary dehydrogenase is used in a second step after the primary enzyme reaction has been arrested. Under these circumstances, the minimal amount of glucose-6-P dehydrogenase required may be calculated from the equation $V = (1/t) \{ (S)_0 + 2.3 K_s \log \{ (S)_0 / (S) \} \}$. V is the activity of the dehydrogenase expressed as velocity with saturating levels of substrate, $(S)_0$ is the highest anticipated initial level of substrate for the auxiliary enzyme, and (S) is the highest permissible level of unchanged substrate after incubation time, t . In the present case, for a 30-minute incubation, and with (S) set at 1% of $(S)_0$, the equation becomes $V = [2(S)_0 + 0.2]$ mmoles per liter per hour, if $(S)_0$ is expressed as millimoles per liter.

In the case of hexokinase, the auxiliary dehydrogenase is present during the first step, and a higher activity is necessary. A simple way to examine the requirements for the auxiliary enzyme is as follows. Let v equal the velocity of either hexokinase or the dehydrogenase (because they must be nearly equal in a valid assay system). Let (S) equal the steady state glucose-6-P concentration that will prevail during most of the reaction, and let t equal the incubation time. (S) is clearly the total error,

whereas vt is the total amount of substrate to be measured. The fractional error = $[(S)/vt]$.

Since (S) will be small compared to K_s (if the error is to be kept small), $V = [vK_s/(S)]$, and therefore $V = [K_s/(\text{fraction error})t]$. For example, if it is desired for an incubation of 30 minutes that TPNH formation be not less than 97% of glucose-6-P formation, and $K_s = 0.02$ (as in the present case), then $V = [0.02/(0.03 \times 0.5)] = 1.3$ mmoles per liter per hour.

The individual methods that follow are designed to measure the activities at nearly optimal conditions of substrate and coenzyme concentration and pH. The conditions are also such as to permit close approximation of proportionality with time and amount of enzyme. Because of the very great tissue dilution, interference from possible soluble inhibitors or accelerators from the tissue seems remote, and no evidence for such interference has been observed.

Hexokinase—The reaction was allowed to take place in the presence of sufficient glucose-6-P dehydrogenase and TPN⁺ to oxidize the glucose-6-P almost as rapidly as it is formed. This is necessary in order to prevent serious product inhibition (6) as well as to obtain stoichiometric TPNH formation.

The samples were incubated for 60 minutes at 38° in 5 μ l of 0.05 M Tris buffer, pH 8.2, containing 8 mM glucose, 5 mM ATP, 5 mM MgCl₂, 15 mM K₂HPO₄, 0.8 mM TPN⁺, 0.05% bovine serum albumin, and sufficient glucose-6-P dehydrogenase to give a maximal velocity of about 2 mmoles per liter per hour. (The reagent without glucose-6-P dehydrogenase and Mg was stored at -20°.) Standards contained 0.06 and 0.2 mM glucose-6-P. After incubation, aliquots of 4 μ l were added to 1 ml of either (a) 0.025 M phosphate buffer, pH 7.4, or (b) 0.02 M Tris buffer, pH 8, containing 0.05 mM TPN⁺, 0.2 mM EDTA³ and sufficient 6-P-gluconate dehydrogenase to catalyze the oxidation of 50% of a low level of 6-P-gluconate (e.g. 0.002 mM) in 5 minutes or less at room temperature. The fluorescence of the samples in phosphate were read as convenient. The fluorescences of the samples with added 6-P-gluconate dehydrogenase were read after 30 minutes at room temperature.

The high dilution in phosphate prevented further hexokinase action. This is in part due to dilution of the substrates, and in part probably due to combination of P_i with Mg. With larger samples, not diluted so much, EDTA can be added to the buffer, as was done in the case of the alternate reagent.

The purpose of the 6-P-gluconate dehydrogenase was not merely to double sensitivity. It was added rather to guard against the possibility that 6-P-gluconate dehydrogenase present in retina might partially oxidize 6-P-gluconate formed in the assay and make results uncertain. The 6-P-gluconate dehydrogenase was prepared from hog liver. A 1:6 homogenate in 0.02 M phosphate buffer, pH 7, containing 0.02 mM EDTA, was fractionated with ammonium sulfate at pH 7. The fraction soluble in 1.6 M and precipitating from 2.3 M ammonium sulfate was refractionated between 1.9 and 2.5 M at a volume of 2 ml per g of original tissue. It was then dialyzed against a solution of the same composition used for making the original homogenate, and treated with protamine sulfate at a concentration of 0.04% at a dilution of 2 ml per g of tissue. The supernatant fluid was heated for 5 minutes at 52°. The soluble remainder was precipitated in 2.6 M ammonium sulfate and refractionated twice more between

² A highly satisfactory preparation of yeast glucose-6-P dehydrogenase is now available from Boehringer and Sons, through California Foundation for Biochemical Research. The kinetics are very similar for this enzyme and for the adrenal enzyme. At 25° and pH 8, the maximal velocity of a 0.5% solution is about 40 moles per liter per hour.

³ The abbreviation used is: EDTA, ethylenediaminetetraacetate.

1.8 and 2.3 M ammonium sulfate at a volume of 0.5 ml per g of original tissue. The activity was about 5 moles per kg of protein per hour at 25° which represented 15-fold enrichment with 20% yield. Glucose-6-P dehydrogenase activity was less than 0.001% of 6-P-gluconate dehydrogenase activity. The preparation, however, was contaminated with substantial amounts of hexokinase, phosphoglucose isomerase, and malic enzyme. Rough measurements at 24° indicated Michaelis constants for TPN^+ of 6×10^{-6} and 7×10^{-6} M at pH 7.9 (0.05 M Tris) and pH 8.9 (0.05 M 2-amino-2-methyl-1,3-propanediol⁴), respectively. Michaelis constants for 6-P-gluconate at 24° were determined to be 2.2, 9.9, and 15.3×10^{-5} M, respectively at pH 7.9 (0.05 M Tris), pH 8.9, and pH 9.2 (0.05 M 2-amino-2-methyl-1,3-propanediol). The maximal velocities had relative values of 1, 0.81, and 0.65 at these same pH values.

Phosphoglucose isomerase—The activity of this enzyme was determined with fructose-6-P as substrate. The product was measured with the aid of glucose-6-P dehydrogenase and TPN^+ . Each sample was incubated with 8 μ l of 0.1 M 2-amino-2-methyl-1,3-propanediol buffer, pH 8.9, containing 0.05% bovine plasma albumin and 4 mM fructose-6-P that had been prepared enzymatically (5). After incubation for 30 minutes at 38°, and chilling in ice water, 3 μ l of 1 N HCl were added. A 10 μ l aliquot was transferred to a fluorometer tube containing 1 ml of 0.1 M Tris buffer, pH 8, with 0.03 mM TPN^+ , 0.5 mM 6-P-gluconate, and sufficient glucose-6-P dehydrogenase to permit oxidation of the standards at a rate of at least 50% in 5 minutes. (The standards were 0.4 mM during the first step or 0.0025 mM during the last step.) After 30 minutes at room temperature, the resulting TPNH fluorescence was read.

Isomerase action on fructose-6-P departs from linearity chiefly due to product inhibition (5). It was empirically determined that if isomerization did not exceed 25% a linear plot could be obtained if the values were corrected as follows:

$$\text{Activity (corrected)} = \text{Activity (observed)} / f,$$

in which $f = 1 - 0.012 \times \% \text{ conversion of fructose-6-P to glucose-6-P}$. The data presented have been corrected in this manner.

The glucose-6-P dehydrogenase preparation used contained a little isomerase. This was inhibited by the addition of 6-P-gluconate (7), which has an inhibitor constant of about 0.005 mM (5). This is about half the value of the Michaelis constant for fructose-6-P (5). Recently available glucose-6-P dehydrogenase preparations² are nearly free of isomerase, and with their use, the 6-P-gluconate is not required. In fact, the analysis in this case can be simplified by incorporating TPN^+ and the dehydrogenase into the first reagent. The only disadvantage of this is that the reaction rate falls off somewhat faster than it does otherwise, because of the competitive inhibition by 6-P-gluconate just mentioned.

The method described for isomerase measurement has substantial advantages in comparison with a method used previously in similar studies (8). The earlier method, like its predecessors, assessed the reaction in the direction of fructose-6-P formation and depended on measurement of color produced with resorcinol in strong acid. The present fluorometric procedure is inherently more sensitive, by a factor of at least 100. It is also more nearly

linear with time because the point of equilibrium is much closer to glucose-6-P than to fructose-6-P, and because the Michaelis constant for fructose-6-P is a third that of glucose-6-P (5). The new procedure has the disadvantage that it is difficult at present to obtain commercially sufficiently pure fructose-6-P. The reaction rate is nearly the same in the two directions with isomerase from a number of sources (5). However, reported analyses with the earlier method are 50% above correct values because of the impurity of commercial fructose-6-P (5) that misled Buell *et al.* (8) as well as earlier investigators.

The procedure described can be readily modified to measure greater or lesser amounts of enzyme under more general circumstances. In this case it is recommended that the analysis be arranged so that the fraction of substrate converted is not less than 2 or 3% (inasmuch as the best fructose-6-P is likely to contain a little glucose-6-P) and not more than 20 or 30% (because of the departure from linearity noted.) To meet this recommendation, it is possible to change not only the incubation volume but also the substrate concentration to any level within the range of 0.2 to 20 mM. (Higher levels have not been tested.) In the case of very low enzyme levels, the sensitivity can be increased by using the indirect procedure for measuring TPNH.

P-fructokinase—This enzyme was measured by a method, adapted from Ling *et al.* (9), in which fructose diphosphate formed is converted with auxiliary enzymes via dihydroxyacetone-P to α -glycerol-P. In the adaptation, the resulting DPN^+ is measured fluorometrically.

The samples were incubated for 30 minutes at 38° in 3 μ l of 0.05 M potassium phosphate buffer, pH 8, containing 10 mM fructose-6-P, 1 mM $(\text{NH}_4)_2\text{HPO}_4$, 5 mM MgCl_2 , 10 mM ATP, 2 mM DPNH, 0.05% bovine plasma albumin, 0.001% crystalline aldolase (Worthington), α -glycerol-P dehydrogenase sufficient to give a velocity of 6 mmoles per liter per hour at 25° with 1 mM dihydroxyacetone-P, and triose phosphate isomerase sufficient to give a velocity of 3 mmoles per liter per hour at 25° starting with 1 mM dihydroxyacetone-P. (The ammonium phosphate is unnecessary. For a better buffered reagent giving comparable enzyme rates, the potassium phosphate may be replaced by 0.1 M Tris, pH 8, and 0.1 M KCl.)

The last two auxiliary enzymes were prepared from rabbit muscle by modifications of published procedures (10, 11).

The reaction was permanently arrested with 3 μ l of 0.5 N HCl, a 5 μ l aliquot was added to 100 μ l of 6 N NaOH in a fluorometer tube. After the sample was heated for 15 minutes at 60°, 1 ml of water was added and the fluorescence measured. Standards consisted of 0.5 mM DPN^+ (equivalent to 0.25 mM fructose-6-P). In addition, 0.3 mM fructose diphosphate working standards were also prepared and incubated for both 5 and 30 minutes to make sure the auxiliary enzymes were sufficiently active.

Glucose-6-P Dehydrogenase—This enzyme was measured in 0.1 M 2-amino-2-methyl-1,3-propanediol at pH 9 to 9.4 containing 2 mM glucose-6-P, 0.5 mM EDTA, 0.05% bovine plasma albumin and 0.3 to 1 mM TPN^+ , depending on the expected enzyme activity. (In some earlier analyses, Mg was added, but was later omitted because it had no apparent effect. In the case of the monkey retina reported in Fig. 2, the reagent also contained 6-P-gluconate dehydrogenase to give a maximal velocity of about 4 mmoles per liter per hour (see below).)

Because of the tremendous range of activity in retinal samples it was necessary to vary the analytical procedure. For example,

⁴ Distillation Products, Inc.

0.05 to 0.1 μg samples from retinal layers 6 to 9 inclusive were incubated for 60 minutes in 2 μl of reagent with 0.5 mM TPN⁺ and supplementary 6-P-gluconate dehydrogenase. The reaction was stopped with 10 μl of 0.25 M Na₃PO₄-0.35 M K₂HPO₄. After being heated 15 minutes at 60°, a 10 μl aliquot was added to 100 μl of 7 N NaOH containing 0.025% H₂O₂ in a fluorometer tube. After being heated for 10 minutes at 60°, 1 ml of water was added and the fluorescence measured. Standards consisted of 0.1 mM TPNH or 6-P-gluconate. On the other hand, samples from layers 2b to 5 inclusive were incubated in 10 μl of reagent with twice the concentration of TPN⁺. After 30 minutes at 38°, 10 μl aliquots were diluted in a fluorometer tube with 1 ml of 0.05 M Na₂CO₃-0.005 M NaHCO₃, and the native fluorescence was read directly.

The supplementary enzyme, 6-P-gluconate dehydrogenase, was added in one series of analyses for the same reason it was used with hexokinase, *i.e.* to make sure that endogenous 6-P-gluconate dehydrogenase did not distort the yield of TPNH. However, the chances for trouble are less than with hexokinase, because at pH 9 the activity of 6-P-gluconate dehydrogenase with optimal substrate is less than at pH 8 (the pH optimum), the final concentration of 6-P-gluconate is usually well below the Michaelis constant (about 0.1 mM at pH 9) and the activity of 6-P-gluconate dehydrogenase is much less than that of glucose-6-P dehydrogenase in the most active retinal layers. Nevertheless, in other analytical situations the use of the supplementary enzyme may be necessary for accurate results.

6-P-Gluconate Dehydrogenase—The samples were added to 5 μl of 0.1 M Tris buffer, pH 8.2, containing 3 mM 6-P-gluconate, 2 mM TPN⁺, 1 mM EDTA. The reagent also contained 0.005% bovine serum albumin, but 0.05% is recommended even though tests showed the same activity for samples analyzed in either concentration of albumin. Layers 2b to 5 inclusive were incubated 30 minutes, the rest 60 minutes, and standards contained 0.01 and 0.025 mM TPNH. The reaction was stopped with 30 μl of 0.25 M Na₃PO₄-0.35 M K₂HPO₄. After being heated for 15 minutes at 60°, 30 μl were added to 100 μl of 7.5 N NaOH with 0.02% H₂O₂. The tubes were heated for 10 minutes at 60° and diluted with 1 ml of water to read.

In rabbit brain, the Michaelis constants for the enzyme at pH 8 are about 0.014 and 0.002 mM for 6-P-gluconate and TPN⁺, respectively. The TPN⁺ excess is not made too great, as it gives fluorescence by the procedure followed, equal to about 1% of an equal amount of TPNH.

Commercial 6-P-gluconate is made from glucose-6-P and may be contaminated with the latter. The contamination must be less than 0.01% for use in the present method, because of the low Michaelis constant of glucose-6-P dehydrogenase. (Thus, 0.1% contamination would have caused a 30% error in some of the retinal analyses.) Recent preparations from Sigma Chemical Company have been entirely satisfactory.

Phosphoglucomutase—The assay conditions used were very similar to those given by Buell *et al.* (8) but instead of measurement of glucose-1-P disappearance, glucose-6-P formation was determined with glucose-6-P dehydrogenase.

The samples were added to 6 μl of 0.01 M Tris buffer, pH 7.6, containing 2 mM glucose-1-P, 2 mM MgCl₂, 0.01 mM glucose-1, 6-diphosphate and 2 mM dimercaptopropanol. After 60 minutes at 38°, 30 μl of 0.01 M Tris buffer, pH 7.6, were added containing 2 mM EDTA, 0.5 mM TPN⁺, sufficient glucose-6-P dehydrogenase to give a maximal velocity of 0.5 mmole per liter per hour at

25°, and sufficient P-glucosomerase to give a maximal velocity of 20 mmoles per liter per hour at 25°. After 15 minutes at 25°, a 30 μl aliquot was diluted in 1 ml 0.01 M phosphate buffer of pH 7.6, and the fluorescence was measured.

Standards consisted of 0.1 mM glucose-6-P. The isomerase (a simple preparation from rabbit muscle (5)) was added in the second step so that any glucose-6-P that might have been converted to fructose-6-P by tissue isomerase in the first step would surely be converted back again during the second, shorter, incubation at greater dilution.

RESULTS

The most striking finding in regard to distribution of *hexokinase*⁵ in both monkey and rabbit is that in the first neuron (receptor cell)⁶ of the retina this enzyme is almost entirely confined to the inner segments of the rods and cones, and in the monkey at least, to the outer part of these segments (Fig. 1, Table I). Incomplete data indicate a similar situation in human retina. The first retinal neuron in both rabbit and monkey is without blood supply. It seems reasonable to suppose that glucose diffuses from the choroidal vessels to the inner segments where it is phosphorylated, and that it diffuses down the neuron to the other end as glucose-6-P. The outer segments contain relatively little hexokinase, but these segments are low in all metabolic enzymes so far measured and may have ample hexokinase for their own purposes.

In the rest of the retina, the fiber or synaptic layers are much richer in hexokinase than the cell body layers. In contrast to the first neuron there is no sign of limitation either to dendrite or axon, because the enzyme in monkey, at least, is high in both a layer of mixed synapses and dendrites, 5b (Fig. 1), and in a layer of axons, 9. The absolute values for hexokinase in the inner layers of the retina are somewhat lower in rabbit than in monkey. These layers (6 to 9 inclusive) are avascular in rabbit but have a rich blood supply in monkey. The low value for hexokinase in pigment epithelium may be noted.

In contrast to the enzyme that *forms* glucose-6-P, the enzymes

⁵ Hexokinase was measured in retinas from three different monkeys. Values for one are shown in Fig. 2, for another in Table II, and a third is not shown. All agreed rather closely except that in the case of the retina represented in Fig. 1, the hexokinase values in the inner layers were lower than for the other two, approximating the values for the rabbit.

⁶ The structure of the retina is drawn schematically on the figures. The pigment epithelium (*layer 1*) lies immediately beneath the choroid. The first neurons, whether rod cells or cone cells, consist of: *outer segments (layer 2a)*, the photoreceptors proper; *inner segments (layer 2b)*, analogous to dendrites; *cell bodies* packed tightly together to make the outer nuclear layer (4); and *naked axons*, making up the outer portion of the outer reticular layer (5a). The first neurons terminate in layer 5b where synapsis is made with the second neurons. These are short bipolar cells with cell bodies lying in the inner nuclear layer (6) together with cell bodies of modified glia (Mueller's cells). The bipolar cells synapse in the inner reticular layer (7) with the third retinal neurons, the ganglion cells, the large cell bodies of which occupy the ganglion cell layer (8) and the fibers (nonmyelinated) of which make up layer 9 as they run toward the optic nerve head. "Outer" is used to mean toward the periphery of the eye ball. The rabbit retina is completely avascular. The monkey retina has a good blood supply extending from the inner (vitreous) surface through layers 9 to 6 inclusive. Layers 1 to 5 have no blood vessels, but the choroid, adjacent to layer 1, has a rich blood supply in both species. The total thickness of the retina is about 0.3 mm in monkey and 0.2 mm in rabbit.

that use this substrate are richest at the distal end of the first cell. *Isomerase* in monkey retina has its peak value in the naked axon portion of the first neuron (Table I). The rabbit retina is similarly constituted to that of the monkey throughout the outer layers, but the inner retinal layers are all rich in isomerase and a second peak is reached in the inner reticular layer. *Phosphofructokinase* (Fig. 1, Table I) tends to parallel isomerase, as one might expect, although the correspondence is far from constant. In the rabbit, the ratio of isomerase to phosphofructokinase only varied over a 2-fold range (Table II), whereas the ratio of hexokinase to phosphofructokinase varied 50-fold. In the monkey, a 5-fold range of isomerase to phosphofructokinase ratios was observed. The relatively high phosphofructokinase value in fiber layer 9 and very low value in cell body layer 6b, with little difference in isomerase activity, was confirmed in another monkey retina.

With respect to the oxidative shunt, it will be seen that glucose-6-P dehydrogenase is exceedingly high throughout the first neuron, except for the outer segment, and even this portion is as rich as average brain (Fig. 2). The peak values for monkey exceed average brain by a factor of 25. Although in the rabbit the values are not as high, the absolute levels are, nevertheless, impressive (Fig. 2). In both species, there are peak values in the innermost portion of the inner segments and in the outer reticular layer, and both show a dramatic decrease in the rest of the retina to levels which are as low as average brain or lower. The next enzyme of the oxidative shunt, 6-P-gluconate dehydrogenase, is also abundant in the first neuron (Table I), although not nearly so active as the preceding enzyme. One might expect close correspondence between these two enzymes

but the ratio of their activities was found to vary over an 18-fold range (Table II).

Still another enzyme acting on glucose-6-P, *phosphoglucosmutase*, is more active in the axonal end of the first neuron than in the inner segment where glucose-6-P is presumed to be originally formed (Table I). This enzyme is quite active in the inner retinal layers, and in these layers it is substantially more abundant in rabbit than in monkey.⁷

DISCUSSION

The retina is a part of the central nervous system serving a specialized function under unusual difficulties. Presumably, in the interests of optical clarity, the outer layers of monkey retina and the full thickness of rabbit retina functions without a penetrating blood supply,⁸ this in spite of very high capacity for oxygen consumption and lactate production. It is, therefore, of interest to see if enzyme distributions among the layers offer any clues as to how the difficulties posed by the anatomical situation have been surmounted.

The finding that hexokinase is localized almost entirely at the end of the first neuron nearest the choroidal blood supply would seem to be an adaptation favorable for function in an avascular zone. Presumably, diffusion would be faster within the cells, because of greater total cross section, than in the tissue spaces. Furthermore, there is the possibility of active motion by the cell or within the cell to hasten transport. If there is a glucose-6-P gradient down the cell, there must be a gradient of some phosphate compound, presumably ATP or P_i in the other direction. Unless the phosphate returns in high energy form, the outer end of the cell must have an exceedingly active ADP phosphorylating system, presumably oxidative. Studies of the distribution of P_i and phosphorylated intermediates might determine the form in which P returns to the inner segment. It might also be useful in this connection to measure the distribution of P-glycerate and pyruvate kinases.

It would appear from the enzyme distributions that the capacity for utilizing phosphorylated hexose in the first retinal cell is greatest at the end furthest from the glucose-6-P supply. The five enzymes studied here that utilize hexose phosphate are all most abundant at the axonal end of this cell. The same is true for lactic dehydrogenase (1), aldolase,⁸ and glyceraldehyde phosphate dehydrogenase (12). The fact that all these members of the glycolytic pathway are very active at the axonal end of the first neuron indicates that the high level of phosphofructokinase is not merely serving to trap fructose-6-P (as an aid to glucose-6-P diffusion down the neuron).

One might speculate as to why in the monkey both ends of the first neuron are not rich in hexokinase, inasmuch as they both come close to a good blood supply. Possibly, this is an evolutionary or embryological carry over, because lower forms, like the rabbit, tend to lack a blood supply throughout the retina and the blood supply to the inner layers when present does not become functional until late in development.

Eichel (13) observed that retina as a whole is rich in glucose-6-P dehydrogenase. This was confirmed by Schimke (14). It is now seen that the abundance of this enzyme is entirely limited

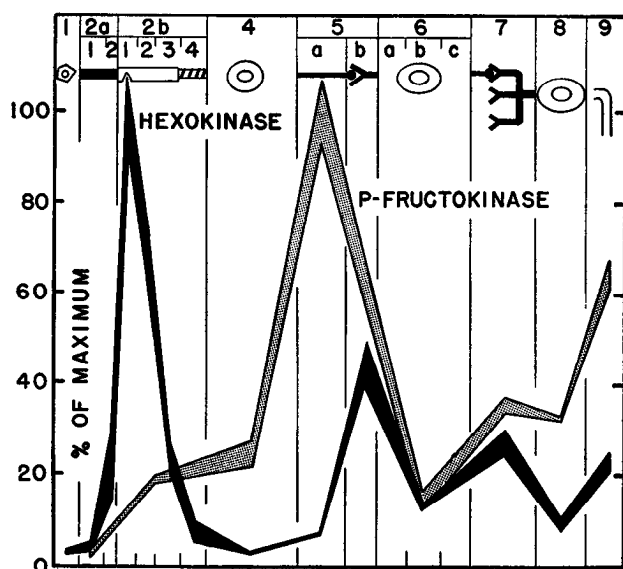


FIG. 1. Distribution of hexokinase and phosphofructokinase in monkey retina. The line widths at the center of each layer equal two standard deviations for that layer. The points have been connected for better visualization, but this does not imply a gradual transition of values from one layer to the next. The layer widths are drawn roughly proportional to actual layer thicknesses. The peak values for hexokinase and phosphofructokinase were, respectively, 27 and 32 moles per kg of fat-free dry weight per hour. The phosphofructokinase data are from retina M 17 (Table I). Hexokinase data are from M 58, except for values at 2a and 5b, which came from M 48 and M 17, respectively.

⁷ Attempts have been unsuccessful to relate the phosphoglucosmutase values of Table I to glycogen as seen by various workers using staining methods.

⁸ Unpublished observations.

TABLE I

Five enzymes in monkey and rabbit retina

Values are moles of substrate transformed per kg of lipid free dry weight per hour at 38°. The individual retinas are identified by a number and M or R for monkey or rabbit. The standard errors are given in italics.

Retinal layer	Hexokinase		Isomerase		Phosphofructokinase		Phosphoglucumutase		6-P-gluconate dehydrogenase
	M48	R59	M48	R60	M17	R54	M48	R59	M58
1 Pigment epithelium	1.4 <i>0.3</i>		35 ^a <i>4</i>			1.2 <i>0.2</i>	2.3 <i>0.1</i>		1.51 <i>0.07</i>
2a Outer segments, rods and cones	1.1 <i>0.3</i>	1.5 <i>0.2</i>	22 <i>2</i>	13 <i>1</i>	0.8 <i>0.2</i>		0.4 <i>0.1</i>	0.8 <i>0.1</i>	0.17 <i>0.08</i>
2b1 Inner segments, rods and cones	21.9 <i>1.9</i>								1.67 <i>0.08</i>
2b2 Inner segments, rods and cones	13.4 <i>1.5</i>	14.2 <i>1.4</i>	71 <i>5</i>	81 <i>1</i>	6.0 <i>0.1</i>	5.7 <i>0.7</i>	3.0 <i>0.3</i>	1.3 <i>0.2</i>	2.42 <i>0.11</i>
2b3 Inner segments, rods and cones	4.8 <i>1.4</i>	1.8 <i>0.1</i>							3.37 <i>0.19</i>
2b4 Inner segments, rods and cones	1.0 <i>0.1</i>	12.4 <i>1.7</i>				12.7 <i>0.8</i>			5.50 <i>0.24</i>
4 Outer nuclear	0.8 <i>0.1</i>	0.2 <i>0.0</i>	122 <i>6</i>	65 <i>5</i>	7.8 <i>0.8</i>	4.2 <i>0.2</i>	5.2 <i>0.2</i>	3.5 <i>0.2</i>	3.07 <i>0.13</i>
5 ^b Outer reticular	3.7 <i>0.6</i>	2.0 <i>0.8</i>	225 <i>8</i>	259 ^c <i>17</i>	31.6 <i>2.2</i>	31.4 <i>0.9</i>	11.2 <i>0.6</i>	12.9 <i>0.7</i>	6.15 <i>0.35</i>
6b Inner nuclear	5.1 <i>0.5</i>	3.6 ^d <i>0.4</i>	105 <i>4</i>	191 <i>19</i>	4.1 <i>0.1</i>	14.9 <i>0.4</i>	7.0 <i>0.3</i>	11.8 ^d <i>0.4</i>	1.37 <i>0.02</i>
7 Inner reticular	15.3 <i>0.6</i>	9.0 <i>1.2</i>	161 <i>12</i>	396 <i>14</i>	11.3 <i>0.5</i>	30.9 <i>0.8</i>	9.2 <i>0.3</i>	13.5 <i>0.4</i>	1.84 <i>0.10</i>
8 Ganglion cell	9.8 <i>0.7</i>	3.6 <i>0.5</i>	75 <i>5</i>	187 <i>21</i>	10.3 <i>0.2</i>	28.2 <i>1.4</i>	7.9 <i>0.3</i>	16.1 <i>0.9</i>	1.54 <i>0.12</i>
9 Fiber	11.8 <i>0.9</i>		95 <i>6</i>	140 <i>12</i>	20.5 <i>0.9</i>	14.4 ^e <i>1.6</i>	10.1 <i>0.7</i>		1.62 <i>0.11</i>
Average rabbit brain		10		140		16		9	1.5

^a Data from monkey M17.

^b Layer 5a only (naked axons) for monkey, full thickness for rabbit except as noted.

^c Outer portion of layer. Inner portion averaged 446 ± 22.

^d Layer 6a plus 6b.

^e Samples were from the bundle of myelinated fibers peculiar to the rabbit.

to the first neuron. It does not seem likely that the significance of this abundance lies in the deficient blood supply to the first neuron, because in rabbit the rest of the retina, which is avascular throughout, is almost as low in glucose-6-P dehydrogenase as in the monkey. It is also difficult to see how this enzyme is related to lipid synthesis, because all portions of this first neuron are unusually low in lipid in comparison with other nervous tissues (1). The unusually high glucose-6-P dehydrogenase activity may be related to the needs of the outer segments, the photoreceptors. As mentioned, these structures are very poor in regard to every metabolic enzyme so far examined. Possibly TPNH or something formed with TPNH is a special substrate for the photoreceptors.⁹

⁹ The distribution of glucose-6-P dehydrogenase shows no obvious relationship to the pattern of deposition of formazan seen by Kuwabara and Cogan (15) when they exposed *intact* retina to tetrazolium with glucose plus TPNH. As the authors suggest, their results may reflect the distribution of enzymes concerned with the dehydrogenation of TPNH rather than the substrate dehydrogenase proper. It also seems possible that substrate and coenzyme may not have been able to penetrate uniformly into the *intact* cells.

Futterman and Kinoshita (16), using glucose-1-C¹⁴ and glucose-6-C¹⁴, could find little evidence for an active direct oxidative pathway in whole retina. They did show that anaerobically in the presence of pyruvate there was a substantial increase in the proportion of CO₂ derived from C₁ of glucose. This suggested to them that the direct oxidative pathway may be present but ordinarily not very active. McIlwain (17) and others have pointed out repeatedly that isolated nervous tissue, unless electrically stimulated, has a lower metabolism than nervous tissue *in situ*. Possibly, the direct oxidative pathway in retina is only called on during nervous activity.

It was seen above that 6-P-gluconate dehydrogenase, although very rich in the first neuron, is not nearly so abundant as glucose-6-P dehydrogenase. It is conceivable that there is an alternate pathway present for utilizing 6-P-gluconate in nervous tissue. McDougal *et al.* (18) have shown that among a large series of fiber tracts, glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase in fact vary inversely. In this connection, it may be mentioned that although Kerly and Rahman (19) found little difference in the proportion of C₁ and C₆ of glucose converted by retina to CO₂, the lactate formed contained considerably more

carbon from C_6 than from C_1 . Thus, C_1 may have been side-tracked without appearing as CO_2 .

The outer layers of the retina of rabbit and monkey correspond rather closely in regard to content of enzymes measured, but the inner layers show marked species differences (Tables I and II). These consist mainly of much higher levels in rabbit of phosphofructokinase, isomerase, and phosphoglucumutase, suggesting greater capacity for glycolysis and glycogen phosphorolysis or synthesis. It has already been reported that this zone contains more lactic dehydrogenase in rabbit than in the monkey (1). It seems reasonable to suppose that this region in the rabbit which is avascular may meet its metabolic needs to a greater degree

through glycolysis than the homologous vascularized region in monkey. Similarly, rabbit retina in an emergency might need to draw more heavily than monkey retina on glycogen stores.

Noell and Chinn (20) discovered that the retina is very susceptible to iodoacetate poisoning, and that this agent has its greatest effect on the outer layers that contain the sensory cells. Not only is function immediately interrupted by iodoacetate, but the outer layers may be completely destroyed without serious damage to the rest of the retina (21, 22). This was first interpreted to mean that the visual cells are more dependent on glycolysis than the cells of the deeper layers. However, it has been reported that oxygen consumption as well as glycolysis may be profoundly depressed in retina by iodoacetate (23, 24), particularly if oxygen consumption is measured in bicarbonate rather than phosphate buffers (23). (The use of bicarbonate markedly increases the respiratory rate.) Similarly, a genetic retinal defect in rat retina, which destroys only the outer layers, results in a nearly parallel loss in rates of glycolysis and oxygen consumption (25). Evidence from the distribution of lactic dehydrogenase (1) as well as that of other enzymes of the glycolytic cycle would suggest that in rabbit (the species first shown to have unusual sensitivity to iodoacetate), the cells lying deeper in the retina should be at least as dependent on glycolysis as the visual cells. Consequently, Noell (26) now suggests that the sensory cells may owe their special iodoacetate sensitivity to the spatial separation of enzymes of glucose metabolism within those cells. The layer of axons (layer 5a) contains exceedingly high levels of the enzymes of glycolysis, including the one most sensitive to iodoacetate, glyceraldehyde-P dehydrogenase (12). Perhaps it is layer 5a that is most susceptible to iodoacetate.

It would be desirable to relate the chemical findings in the retina to the cytological structure. As seen in the electron microscope the outer segments, the photoreceptors proper consist of a pile of disks (27) or flattened sacs (28) with little sign of either mitochondria or smaller particles. Apparently, these specialized structures are able to function with very low

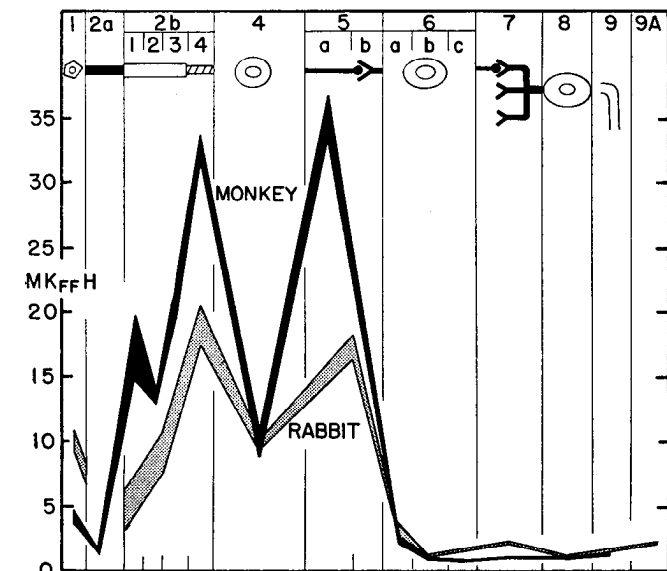


FIG. 2. Distribution of glucose-6-P dehydrogenase in monkey and rabbit retina. The line widths have the same significance as in Fig. 1. Activities are recorded as moles of substrate oxidized per kg of fat-free dry weight per hour (38°). The monkey retina is M 58 of Table I and Fig. 1. The rabbit retina is R 54 of Table I.

TABLE II
Ratios of enzyme activities in retina

Layer	Hexokinase Phosphofructokinase		Phosphoglucosomerase Phosphofructokinase		Glucose-6-P dehydrogenase Hexokinase		Glucose-6-P dehydrogenase Phosphofructokinase		Phosphoglucumutase Phosphofructokinase		Glucose-6-P dehydrogenase 6-P-Gluconate dehydrogenase
	M	R	M	R	M	R	M	R	M	R	M
1					3.0						1.4
2a	1.4		27		1.3		1.8		0.50		8.5
2b	1.5	2.5	12	14	1.9 ^a	0.6	2.9	1.6	0.50	0.23	5.9 ^b
4	0.10	0.05	16	16	12	49	1.3	2.4	0.67	0.83	3.2
5 ^c	0.12	0.06	7	11	8	9	1.1	0.55	0.35	0.41	5.1
6b	1.2	0.24	26	13	0.18	0.31	0.23	0.08	1.70	0.79	0.68
7	1.4	0.29	14	13	0.07	0.25	0.09	0.08	0.81	0.44	0.57
8	1.0	0.13	7	7	0.10	0.30	0.10	0.04	0.77	0.57	0.67
9	0.6		5		0.11		0.07		0.49		0.83
Rabbit brain		0.7		9		0.15		0.09		0.56	1.0

^a The sublayers 2b1, 2b2, 2b3, and 2b4 gave ratios of 0.8, 1.0, 4.3, and 33, respectively.

^b The sublayers 2b1, 2b2, 2b3, and 2b4 gave ratios of 10.3, 5.6, 6.2, and 5.9, respectively.

^c Layer 5a only (naked axons) for monkey, full thickness for rabbit.

levels of the enzymes measured to date. Except for glucose-6-P dehydrogenase no values were greater than 15% of those of average brain. The inner segments of the rods and cones contain, in the outer portion where hexokinase is most concentrated, a dense collection of large mitochondria. It seems probable that hexokinase is associated with these mitochondria, although there are also present minor collections of smaller particles in the inner segment (28). Hexokinase of the central nervous system is insoluble (5). The nuclei of large sensory cells contain hexokinase although at half the concentration of the cytoplasm.¹⁰ In the retina the rod nuclei must be practically devoid of hexokinase.

The remainder of the outer neuron (fibers, fluid around the nucleus, and synaptic terminal) contains almost no solid particles visible in the electron microscope except for neuroprotofibrils and synaptic vesicles (29). The distribution of glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase is that expected if they were evenly distributed in the cytoplasm, but excluded from the formed elements, including the nuclei. It will be noted that within the inner segment itself there is a reciprocal relationship between glucose-6-P dehydrogenase and hexokinase (Figs. 1 and 2) *i.e.* the mitochondria in the outermost portion leave little room for cell sap. The high relative concentration of isomerase, phosphoglucumutase, and phosphofructokinase in the outer reticular layer suggests association with nondiffusible elements, possibly the synaptic vesicles. This is surprising, as these enzymes are found in the supernatant fluid of tissue homogenates fractionated by centrifugation.

In the rest of the retina, it will be noted that the layers containing cell bodies and their nuclei tend to contain lower concentrations of all the enzymes measured than the reticular and fiber layers.

SUMMARY

1. Six enzymes concerned with glucose metabolism have been measured in histologically pure samples of 12 layers or sublayers of retina in monkey and rabbit.

2. Hexokinase within the first neuron of the retina is almost entirely confined to the inner segments of the rods and cones. Phosphoglucumutase, phosphoglucoisomerase, and phosphofructokinase are relatively concentrated at the opposite end of the cell.

3. Glucose 6-phosphate dehydrogenase is exceedingly rich throughout most of the first neuron. Peak values are 10 to 30 times those of average brain or of the rest of the retina. Parallel but less marked differences were found in the case of 6-phosphogluconate dehydrogenase.

¹⁰ Unpublished results.

4. The outer segments of the rods and cones are very deficient in all the enzymes measured.

5. The deeper retinal layers, containing the second and third neurons, are richer in rabbit than monkey in regard to phosphoglucumutase, phosphoglucoisomerase, and phosphofructokinase. This species difference is associated with a difference in blood supply.

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