The metabolism of retinal pigment cell epithelium

II. Respiration and glycolysis

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A study of respiration and glycolysis in vitro of retinal pigment epithelium and retina minus the pigment epithelial layer has indicated metabolic similarities and differences between the two tissues. Aerobic lactic acid production from glucose was one-half in pigment epithelium what it was in retina, and the Pasteur effect operated with approximately the same efficiency in either tissue. Pigment epithelium, like retina, showed no evidence of an active phosphogluconate pathway under the experimental conditions used. On the other hand, despite evidence for a functional tricarboxylic acid cycle in pigment epithelium, glycolysis considerably predominated over oxidation in the catabolism of exogenous glucose by this tissue. Also different from what was found for retina was the observation that there was no CO₂ fixation in pigment epithelium.

The pigment cell epithelium is a vital layer of the retina. Separation of the neural part of the retina from the pigment epithelial layer, as occurs in retinal detachment, is accompanied by an immediate loss of vision. Degenerative disease of the pigment epithelium leads to deterioration of the visual cells.

Observations on the fine structure of the retinal pigment epithelium and its anatomical relationship to the visual cell outer segments and the choroid suggest that the pigment epithelium mediates the transport of metabolites from the choriocapillaris to the visual cells. Other studies have indicated that the pigment epithelium contributes directly to the visual cycle by esterifying, storing, and possibly isomerizing vitamin A alcohol. The energy requirements of these functions are not known. Indeed, the only quantitative information about the energy metabolism of retinal pigment epithelium derives from two investigations: one concerning its content of metabolic enzymes and the other its incorporation of ³²P in vitro.

Knowledge about the general metabolic pathways in pigment epithelium can lead to a better understanding of its function in normal and impaired vision. This paper concerns a study of the respiration and glycolysis of bovine retinal pigment epithelium in vitro as compared with that of retina minus the pigment epithelial layer.

Methods

Beef eyes were brought quickly on ice from a local slaughterhouse. Retinas were dissected and pigment epithelium was brushed from the choroidal...
surface of the eyecup as previously described. The pooled suspension, in cold Ringer’s solution, of pigment epithelial cells from 30 to 50 eyes was centrifuged for 5 minutes at low speed, the supernatant was discarded, and the cells were resuspended in fresh Ringer’s solution such that 2 ml. samples contained pigment cells from 5 to 8 eyes (equivalent to 4 to 7 mg. protein). The samples were incubated at 38° C. for estimation of respiration and glycolysis. Parallel determinations were made on fragments, usually quarters, of intact retina in 2 ml. medium.

Krebs-Ringer solutions buffered to pH 7.4 with tris, phosphate, or bicarbonate were used for preparation and incubation of the tissues. The Ringer-tris and Ringer-bicarbonate solutions contained 2 mM. phosphate.

Oxygen uptake was determined by the direct method of Warburg. At the end of the incubation period 0.5 ml. of 30 per cent trichloroacetic acid was added to each sample, and the samples were transferred quantitatively into centrifuge tubes. After centrifugation, the supernatant was analyzed for lactic acid and the residue for protein. Glycogen was determined by the method of Kahan.

For investigation of the metabolism of 14C-labeled glucose, the tissue samples were incubated in a shaking water bath in 25 ml. Erlenmeyer flasks having glass center wells and rubber serum bottle stoppers. For convenient collection of CO2, each center well was fitted with a removable, thin glass, open-mouthed vial which contained 0.15 ml. Hyamine hydroxide solution (1 M in methanol) and a filter paper wick. At the end of the incubation period the trichloroacetic acid was added to the tissue suspensions by injection through the rubber stoppers, and the flasks were shaken an additional 30 to 60 minutes to insure complete collection of CO2. When the Ringer-bicarbonate solution was used and the flasks were gassed with 95 per cent O2-5 per cent CO2 at the beginning of the experiment, the Hyamine hydroxide solution was not added until after the incubation period when it was injected through the rubber stoppers into the center-well inserts immediately before the addition of trichloroacetic acid to the tissue suspension.

The center-well inserts were transferred to scintillation counting vials where they were flushed out with dioxane-naphthalene scintillation fluid by means of individual disposable pipettes. The inserts, with their filter paper wicks, were left in the counting vials which were filled to a volume of 12 ml. with scintillation fluid. The samples were counted in a Packard Tricarb automatic scintillation spectrometer.

Radioactivity of the lactic acid was determined after removal of radioactive glucose and other contaminants by chromatography on Dowex-1 formate or by the CuSO4-Ca(OH)2 precipitation step of the Barker and Summerson procedure. Aliquots (usually 0.5 ml.) of the appropriate fraction of eluate or the supernatant, respectively, were counted. Comparable results were obtained by either method.

Results

Fig. 1 shows the endogenous respiration of pigment epithelium, compared with that of retina, and the effect of glucose or succinate on the respiratory rates. The respiration of either tissue was fairly linear during 2 hours regardless of substrate. The endogenous respiration of pigment epithelium was significantly increased by succinate (p < 0.001), and that of retina was increased by succinate or glucose. The endogenous and succinate-stimulated rates of oxygen uptake in pigment epithelium were about one-third the corresponding rates in retina.

Although there was no significant difference between the mean respiratory rates of pigment epithelium in glucose and substrate-free medium, as illustrated in Fig. 1, such differences were consistently found within individual experiments. Within each experiment the variation among replicate QO2 determinations was considerably less than the over-all variation for the whole series (Table I), and differences due to substrate, although small, were significant. Thus, the mean of twelve individually obtained differences between the QO2 values for pigment epithelium in glucose and substrate-free media was 0.81 ± 0.35. Similarly, the mean of nine individual differences between retinal QO2 values in succinate and glucose media was 3.95 ± 0.86. According to Fisher’s null hypothesis, each of the values is highly significant (p < 0.001). This suggests, therefore, that pigment epithelial respiration was stimulated by glucose and that succinate did have a greater effect than glucose on retinal respiration.

The marked stimulation of pigment epithelial respiration by succinate (Fig. 1) suggested that the tricarboxylic acid cycle was active in this tissue. It was of interest to determine whether glucose oxidation in
Fig. 1. Respiration in air of retinal pigment epithelium and intact retina in Ringer-tris solution without added substrate (E) or containing 8.33 mM glucose (G) or sodium succinate (S). The vertical lines indicate ± one standard deviation. The numbers of determinations are shown in parentheses.

Table I. A comparison of the reproducibility of QO₂* values for pigment epithelium and retina within individual experiments as compared with the over-all mean derived from several experiments

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pigment epithelium</th>
<th>Retina</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Entire series (mean QO₂ ± 1 S.D.)</td>
<td>Individual experiments (mean QO₂ ± 1 S.D.)</td>
</tr>
<tr>
<td>None</td>
<td>2.14 ± 0.62 (23)</td>
<td>1.59 ± 0.16 (3)</td>
</tr>
<tr>
<td></td>
<td>2.32 ± 0.16 (3)</td>
<td>2.80 ± 0.18 (3)</td>
</tr>
<tr>
<td></td>
<td>1.74 ± 0.15 (4)</td>
<td>11.35 ± 1.38 (38)</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.62 ± 0.72 (44)</td>
<td>3.37 ± 0.15 (4)</td>
</tr>
<tr>
<td></td>
<td>2.73 ± 0.18 (3)</td>
<td>4.78 ± 0.25 (3)</td>
</tr>
<tr>
<td></td>
<td>5.72 ± 0.25 (3)</td>
<td>6.37 ± 0.23 (3)</td>
</tr>
<tr>
<td>Succinate</td>
<td>5.52 ± 0.82 (20)</td>
<td>14.39 ± 1.35 (16)</td>
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The data from individual experiments, as listed in this table, were chosen from a group of seven to eight experiments in order to illustrate the complete range of values found. The individual experiments chosen for retina and pigment epithelium were not necessarily the same. There was no parallel or reciprocal relationship between the extreme values obtained for the two tissues. The standard deviations of the mean values for the other experiments were identical with those of the examples. The determinations were made in air in Ringer-tris solution. Substrate concentration was 8.33 mM. The numbers of determinations are shown in parentheses.

*all QO₂ consumed per milligram protein during the first hour.
Table II. The metabolism of 1-14C-glucose and 6-14C-glucose in pigment epithelium and retina

<table>
<thead>
<tr>
<th></th>
<th>14C-glucose</th>
<th>614C-glucose</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Lactic acid</td>
</tr>
<tr>
<td><strong>Tris experiments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigment epithelium (10)</td>
<td>2.9 ± 0.7*</td>
<td>127.1 ± 14.9</td>
</tr>
<tr>
<td>Retina (10)</td>
<td>17.9 ± 4.6</td>
<td>248.8 ± 47.0</td>
</tr>
<tr>
<td>Corneal epithelium (7)</td>
<td>2.4 ± 0.4</td>
<td>45.3 ± 3.6</td>
</tr>
<tr>
<td><strong>Bicarbonate experiments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigment epithelium (4)</td>
<td>12.6 ± 2.6</td>
<td>193.0 ± 10.8</td>
</tr>
<tr>
<td>Retina (4)</td>
<td>76.9 ± 8.7</td>
<td>731.5 ± 100.8</td>
</tr>
<tr>
<td>Corneal epithelium (4)</td>
<td>11.6 ± 1.1</td>
<td>61.8 ± 4.7</td>
</tr>
</tbody>
</table>

Tris experiments: Samples were incubated 2 hours in air in Ringer-tris solution containing 8.33 mM glucose with a specific activity of 3,000 counts per minute per /imole.
Bicarbonate experiments: Samples were incubated for 3 hours in 95 per cent O<sub>2</sub> — 5 per cent CO<sub>2</sub> in Ringer-bicarbonate solution containing 20 m. glucose with a specific activity of 3,000 counts per minute per /imole. The numbers of determinations are shown in parentheses.

*Standard deviation.

pigment epithelium took place by way of the tricarboxylic acid cycle or the phosphogluconate pathway. The relative importance of the two pathways was estimated in pigment epithelium, retina, and corneal epithelium by comparing the metabolism of 1-14C-glucose and 6-14C-glucose (Table II).

There was no significant difference between the mean amounts of CO<sub>2</sub> produced from the 1-carbon and 6-carbon of glucose by pigment epithelium in tris medium, nor was the mean difference between paired samples significant. In retina, also, there was no significant difference between the rates of oxidation of the 1-carbon and 6-carbon of glucose, although the amount of glucose carbon oxidized was considerably greater than what was observed for pigment epithelium. Corneal epithelium, on the other hand, produced the same amount of 14CO<sub>2</sub> from 1-14C-glucose as pigment epithelium, but only one-sixth as much 14CO<sub>2</sub> from 6-14C-glucose.

The metabolism of labeled glucose was also studied in bicarbonate medium for more direct comparison of the 1-14C/6-14C ratios for corneal epithelium and retina with those reported by other investigators. The quantitative effect of bicarbonate on glucose metabolism could not be ascertained from these data (Table II) because the time, glucose concentration, and oxygen tension were different from those used in the experiments with tris medium. However, the respective 1-14C/6-14C ratios for CO<sub>2</sub> production by each tissue were identical in either medium, and the ratios for corneal epithelium and retina agreed with those in the literature.

For all three tissues the 1-14C/6-14C ratios for lactic acid produced were the same, about unity regardless of the medium used. Kinoshita and co-workers obtained a ratio of 0.65 for corneal epithelium. Rahman and Kerly obtained a ratio of 0.75 for retina; however, Futterman and Kinoshita's data gave a ratio of 0.89 for retina, which is closer to the value we obtained. The reason for the discrepancy between our results and those of the other investigators is not clear; it may reflect the influence of experimental variables on triose metabolism. Katz and Wood have pointed out that despite a constant proportion between glycolysis and the phosphogluconate pathway, the ratio for the production of radioactive triose phosphate from 1-14C-glucose and 6-14C-glucose can vary because of quantitative changes in the pathways of glucose metabolism which do not include triose phosphate as an intermediate. It is probable that a variability in triose phos-
phate production would lead to parallel changes in lactic acid production.

The ratio of the yields of $^{14}$CO$_2$ from L-1$^{14}$C-glucose and 6-1$^{14}$C-glucose is also influenced by complex metabolic variables which preclude quantitative evaluation. It would appear qualitatively from the $^{14}$CO$_2$ data in Table II, however, that corneal epithelium was the only one of the three ocular tissues studied which showed any evidence of the direct oxidation of glucose. Pigment epithelium, like retina, did not oxidize glucose by the phosphogluconate pathway within the experimental conditions described.

Although respiration in pigment epithelium was low relative to the retina, aerobic glycolysis was considerable (Table III). In glucose-tris medium pigment epithelium produced almost half as much lactic acid as did retina. Lactic acid production by either tissue was small and variable in absence of substrate or in presence of succinate. This observation was consistent with the detection of only a small amount of glycogen in three representative samples of pigment epithelium and retina. The mean values were 14.5 and 12.8 µg glycogen per milligram protein, respectively (9 and 7 µg per milligram dry weight, respectively).

When the lactic acid production and oxygen consumption in glucose-tris medium were corrected for endogenous metabolism and converted into net equivalents of glucose utilized (Table III), it became apparent that pigment epithelium glycolyzed one-half as much, but oxidized only one-twentieth as much glucose as did retina. This predominance of glycolysis over glucose oxidation in pigment epithelium, as compared with retina, was also evident in the experiments with radioactive glucose (Table II), although here the difference between the tissues was not as great as that which was calculated from the nonradioactive data in Table III.

When the influence of environmental changes on glycolysis was examined, other differences between pigment epithelium and retina were observed (Fig. 2). In Ringer-tris-glucose solution, lactic acid production by either tissue in oxygen was not significantly different from that in air, and glycolysis of both tissues increased in nitrogen. However, the anaerobic increase

### Table III. Glycolysis and respiration of pigment epithelium and retina in air in Ringer-tris solution

<table>
<thead>
<tr>
<th>Time</th>
<th>Substrate</th>
<th>Lactic acid produced (nmoles/Gm. protein ± 1 S.D.)</th>
<th>Oxygen consumed (nmoles/Gm. protein ± 1 S.D.)</th>
<th>Glucose glycolyzed glucose oxidized (equivalents)</th>
<th>Lactic acid produced (nmoles/Gm. protein ± 1 S.D.)</th>
<th>Oxygen consumed (nmoles/Gm. protein ± 1 S.D.)</th>
<th>Glucose glycolyzed glucose oxidized (equivalents)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr.</td>
<td>None</td>
<td>96 ± 27 (23)</td>
<td></td>
<td></td>
<td>331 ± 38 (16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>242 ± 59 (15)</td>
<td>107 ± 32 (51)</td>
<td>$\frac{121}{4} = 30.6$</td>
<td>679 ± 97 (15)</td>
<td>577 ± 62 (42)</td>
<td>$\frac{340}{82} = 4.1$</td>
</tr>
<tr>
<td>2 hr.</td>
<td>None</td>
<td>22 ± 20 (7)</td>
<td>215 ± 47 (23)</td>
<td>47 ± 44 (10)</td>
<td>89 ± 80 (7)</td>
<td>1,130 ± 125 (15)</td>
<td>933 ± 67 (15)</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>72 ± 70 (5)</td>
<td>439 ± 54 (20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>412 ± 83 (44)</td>
<td>237 ± 67 (44)</td>
<td>$\frac{195}{7} = 27.9$</td>
<td>861 ± 102 (20)</td>
<td>927 ± 113 (38)</td>
<td>$\frac{407}{131} = 3.1$</td>
</tr>
</tbody>
</table>

Substrate concentration was 8.33 mM. The numbers of determinations are shown in parentheses.
(Pasteur effect) seemed to be proportionally greater in retina (63 per cent, p < 0.001) than in pigment epithelium (40 per cent, p < 0.01). Neither aerobic nor anaerobic glycolysis of pigment epithelium was affected by changing the medium from Ringer-tris to Ringer-phosphate solution, but lactic acid production by retina increased slightly in the phosphate medium (p < 0.02) although the proportion between aerobic and anaerobic glycolysis was the same as that in Ringer-tris solution.

The most marked differences between pigment epithelial and retinal glycolysis could be observed when the tissues were incubated in Ringer's bicarbonate solution. It is well known that lactic acid production by retina is greatly increased in bicarbonate medium. This phenomenon was clearly indicated by the data in Fig. 2. Aerobic glycolysis of retina was double in bicarbonate medium what it was in phosphate medium, and the increase in anaerobic glycolysis was even greater. Furthermore, in Ringer-bicarbonate solution, but not in the other media, there was a small difference (p < 0.02) apparent between the yield of lactic acid in air and in oxygen.

By contrast, aerobic glycolysis of pigment epithelium was no different in bicarbonate medium from that observed in the other media. Anaerobic glycolysis of pigment epithelium was increased in bicarbonate medium; however, this increase was 70 per cent greater than that found in the other media, whereas the corresponding increase in retinal anaerobic glycolysis in bicarbonate medium was 250 to 300 per cent.

The amount of lactic acid produced by retina in tris or phosphate medium was twice the amount produced by pigment epithelium. This ratio obtained in nitrogen as well as in air and oxygen (Table IV) which signifies that the Pasteur effect was of similar magnitude, proportionally, in the two tissues. In bicarbonate medium retinal glycolysis was more than four times that of pigment epithelium regardless of the atmosphere. Here, again, the increase in glycolysis by nitrogen was proportionally the same in the two tissues; however, pigment epithelial glycolysis was not stimulated by

<table>
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<tr>
<th>Medium</th>
<th>Oxygen</th>
<th>Air</th>
<th>Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>2.1</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Phosphate</td>
<td>2.5</td>
<td>2.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>4.2</td>
<td>4.6</td>
<td>4.6</td>
</tr>
</tbody>
</table>

A comparison of the relative production of lactic acid by pigment epithelium and retina in medium containing 8.33 mM. glucose. The atmosphere used with Ringer-bicarbonate solution contained 5 per cent carbon dioxide.

Fig. 2. The effect of experimental environment on the glycolysis of pigment epithelium and retina. Glucose concentration was 150 mg. per 100 ml. (8.33 mM.). In the experiments with bicarbonate medium the atmosphere contained 5 per cent carbon dioxide. The vertical lines indicate ± one standard deviation. The number of determinations is shown above each bar.
bicarbonate as was retinal glycolysis. Ratios similar to these could be calculated from the data in Table II for production of radioactive lactic acid by the two tissues in tris and bicarbonate medium.

Discussion

The observations from these experiments substantiate what has already been published about the in vitro metabolism of bovine retina minus the pigment epithelium.\textsuperscript{15, 16} Retina has a very high rate of endogenous respiration which is stimulated by glucose and succinate. Glucose oxidation under usual experimental conditions apparently takes place by way of the tricarboxylic acid cycle rather than the phosphogluconate pathway. Retina produces considerable lactic acid aerobically, and even more anaerobically. Moreover, both aerobic and anaerobic rates of glycolysis are greatly increased in bicarbonate medium, presumably by CO₂ fixation.

Compared with retina, pigment epithelium has a low rate of endogenous respiration and oxidizes little glucose; as in retina, however, glucose oxidation is apparently by way of the tricarboxylic acid cycle. The predominant aerobic pathway of glucose catabolism in pigment epithelium appears to be glycolysis. The rate of aerobic glycolysis in this tissue is one-half that in retina and is considerably greater than that in other tissues. The Pasteur effect in pigment epithelium and retina is proportionally similar regardless of the phosphate or bicarbonate concentration of the medium. On the other hand, the increase observed in the Pasteur effect in pigment epithelium in bicarbonate medium over that in the other media is probably caused by increased efficiency of the Pasteur effect by a mechanism other than CO₂ fixation because there is no other evidence that CO₂ fixation occurs in pigment epithelium as it does in retina.

It is logical to compare the metabolism of pigment epithelium with that of the visual part of the retina. Pigment epithelium, a single layer of uniform and relatively uncomplex cells, could be expected to require less energy for maintenance of its membranes and other cellular structures than would the rest of the retina with its heterogeneous mixture of cells and intricately enmeshed and interrelated cell boundaries. Furthermore, the transportation of vitamin A and other substances across the cell membranes of pigment epithelium, although undoubtedly an endergonic process, would predictably require less energy than would be needed by the neural part of the retina for amplification and transmission of electrophysiologic impulses. Indeed, the neural part of the retina has the highest rate of metabolism of any normal tissue.

What is surprising about pigment epithelial metabolism is that glycolysis appears to be the predominant energy-producing pathway despite the fact that there is a functional tricarboxylic acid cycle which can be stimulated by exogenous succinate. Furthermore, an abundant supply of oxy-
gen is available to this layer of cells in vivo from the choriocapillaris.

Retinal pigment epithelium begins as a single layer of neural ectoderm which develops early into a single layer of identical cells and does not differentiate further in the normal mammalian eye. It is possible that the prominent aerobic glycolysis of pigment epithelium represents the retention of a portion of its embryonic metabolic pattern. It is possible also that the usual energy requirements of this tissue can be adequately met by glycolysis and that respiratory mechanisms, although present, act only in reserve. Futterman has shown that the oxidation of glucose by glucose-6-phosphate dehydrogenase is stimulated by pyruvate in whole retina (minus the pigment epithelium) and, to some extent, in isolated rod outer segments. It will be interesting to see whether this phenomenon is found also in pigment epithelium. Such experiments are planned.

Another possibility which must be considered is that the usual exogenous substrate for pigment epithelial respiration is some metabolite other than glucose. We found that glucose oxidation in pigment epithelium was small compared with the oxidation of endogenous substrate or exogenous succinate. Lowry showed that, in comparison with their relative activity in the rest of the retina, the activity of hexokinase, lactic dehydrogenase, and glucose-6-phosphate dehydrogenase in pigment epithelium was much lower than that of other enzymes investigated. Also, the substrate turnover values for the three above-named enzymes in pigment epithelium were considerably smaller than those found for malic dehydrogenase in this tissue. It would seem, therefore, that pigment epithelial respiration is maintained by a substrate which enters the tricarboxylic acid cycle directly. Glutamate is a logical choice, but preliminary experiments have shown that sodium glutamate does not stimulate respiration in pigment epithelium at all, although it has a large effect on retinal respiration.

The possibilities mentioned must be thoroughly investigated in order to characterize more completely the metabolic pathways of pigment epithelium. Such experiments are in progress. However, from what has been discussed in this paper it is clear that pigment epithelium has an energy metabolism which, despite certain similarities, is quite distinct from that of the neural part of the retina.

We gratefully acknowledge the valuable technical assistance by Mrs. Mary Hayward, Mrs. Danuta Fast, and Mr. Cornelius Young.

REFERENCES


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The role of reduced triphosphopyridine nucleotide in the visual cycle, J. Biol. Chem. 238: 1145, 1963.

Discussion

Dr. A. Franceschetti, Geneva, Switzerland. In 1962 Mawas (Bull. Acad. nat. med. 146: 565, 1962) stressed the fact that the activity of the pigment epithelium cell of the retina is not limited to pigment and purple production, but that the presence of mitochondria, secretion grains, and the important activity of the nucleus permit to suppose that it plays an important role for the nutrition of the retina.

With nitro blue tetrazolium (method of Pearse), it is possible to demonstrate several dehydrogenases in the retinal pigment cell epithelium of albino rats, especially those concerning the glycolysis (lactate dehydrogenase activity [Fig. 1], a-glycerophosphate dehydrogenase, DPN-diaphorase).

Fig. 1. Albino rat. Demonstration of lactate dehydrogenase in pigmentary epithelium of the retina. (Pearse nitro blue tetrazolium stain.)