Table I. De-salted PFK-II (see Fig. 2 for preparation) was diluted with an equal volume of 0.2 M Tris-Cl (pH 6.8) containing an effector (final concentration indicated), and incubated at 37°C for 10 minutes to test the thermoprotection of the effectors.

<table>
<thead>
<tr>
<th>Effector (mM)</th>
<th>PFK activity</th>
<th>Initial</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (5)</td>
<td></td>
<td>8.08</td>
<td>9.92</td>
</tr>
<tr>
<td>ADP (1)</td>
<td></td>
<td>5.56</td>
<td>3.34</td>
</tr>
<tr>
<td>AMP (0.5)</td>
<td></td>
<td>4.60</td>
<td>0.70</td>
</tr>
<tr>
<td>Fru-6-P (5)</td>
<td></td>
<td>5.76</td>
<td>6.60</td>
</tr>
<tr>
<td>Na₂SO₄ (10)</td>
<td></td>
<td>5.44</td>
<td>5.86</td>
</tr>
<tr>
<td>Pi (10)</td>
<td></td>
<td>6.40</td>
<td>4.40</td>
</tr>
<tr>
<td>KCl (50)</td>
<td></td>
<td>6.00</td>
<td>0.00</td>
</tr>
<tr>
<td>No addition</td>
<td></td>
<td>7.00</td>
<td>0.60</td>
</tr>
</tbody>
</table>

ATP thus appears to function as a substrate, an inhibitor, and a stabilizer for lens PFK, while sulfate ions de-inhibit the ATP inhibitory effect and also stabilize the enzyme against thermoinactivation at neutral pH or above. These functions are probably operable in the lens under physiologic conditions.

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From the Howe Laboratory of Ophthalmology, Harvard Medical School, and the Massachusetts Eye and Ear Infirmary, Boston, Mass. This project was supported by Grants Nos. EY00089 and EY01276, awarded by the National Eye Institute, Department of Health, Education, and Welfare. Submitted for publication Nov. 18, 1975. Reprint requests: Dr. H. M. Cheng, Howe Laboratory of Ophthalmology, 243 Charles St., Boston, Mass. 02114.

Key words: phosphofructokinase, rat lens, pH-dependent properties, ATP and sulfate protection, cold lability, thermolability, physiologic conditions.

REFERENCES

On the presence of bilirubin in the ocular humors of premature infants. Richard B. KURZEL* and Robert L. HEINRIKSON.

The yellow pigments observed in the ocular humors of premature infants were identified as
the plasma pigments bilirubin and oxyhemoglobin. The blood/vitreous humor barrier to bilirubin was estimated to be (1/0.11) for total bilirubin and (1/0.25) for direct-acting bilirubin. Hall method histochemical staining for bilirubin in sections of premature infant eyes was highly positive for the vitreous humor, and suggestive of uptake of bilirubin by the neural-retinal cells.

In this communication, we report the presence of bilirubin in the ocular humors in all states of hyperbilirubinemia, and in particular, in the eyes of premature infants. We have identified the blood pigments bilirubin and oxyhemoglobin in the vitreous humor by various techniques, and have quantified their presence, making a rough estimate of the blood/vitreous humor barrier to bilirubin.

Experimental. Samples used in this work were collected over a one-year period from infants delivered at the Chicago Lying-In Hospital. Cord blood was obtained at the time of birth, and eyes were obtained at autopsy from stillborn premature infants and premature infants dying within the first few hours of life. Eyes were taken from 17 premature infants (30 to 35 weeks of gestation), with one stillborn infant in the sampling being 24 weeks of gestation. The eyes from three infants dying in their first week of life were also studied, as were the eyes of one adult (age 65 years) with hyperbilirubinemia secondary to liver disease. The eyes of three children and two adults without hyperbilirubinemia were studied for comparison. The cause of death of most of the premature infants was respiratory distress syndrome. None of the infants studied suffered from erythroblastosis fetalis (none available). The eyes were removed from the deceased no later than four to eight hours postmortem. Removal of the eyes immediately after death was unfortunately impossible due to legal considerations (obtainment of consent). The vitreous humor was extracted from the globe with a suction dropper, after making a corneal incision and removing the aqueous humor and lens. The vitreous humor and serum from the cord blood were stored separately in opaque containers shielded from light in order to prevent photo-oxidation and photo-decomposition of the contents. They were stored in a freezer at -10° C. until ready to be studied. From each individual sample, the vitreous humor was separated from the fibrous matrix of the vitreous body and the ora serrata collarette by ultracentrifugation at 10,000 × g. The spectral characteristics of the vitreous humor were analyzed using a Cary 14 spectrophotometer. The spectrophotometric analysis was repeated on vitreous humor exposed to room air and diffuse sunlight for a period of several hours. Individual vitreous and serum samples were analyzed for chemical confirmation of the spectrophotometric pigment identification by the Jendrassik method for bilirubin determination, and by the benzidine method of Crosby and Furth for the determination of oxyhemoglobin.

Histology. The eye of one premature infant was studied histochemically. The fixation of the tissue, method of embedding, and staining by the Hall method for bilirubin determination were carried out by the procedure of Luna. Prior to processing, a small corneal incision was made, and the tissue was fixed in 2 per cent glutaraldehyde/phosphate buffer (pH 7.4, 0.1 M) for 48 hours at 6° C, with three buffer changes. Unstained and hematoxylin and eosin (H & E) stained sections were also produced. Bilirubin staining of the vitreous humor and retina were studied with a Zeiss optical microscope under ×400 magnification. Unstained sections were studied microscopically using a Zeiss No. 4720 interference filter under the stated magnification. The absorption spectrum of all patients in a hyperbilirubinemic state were found to be pigmented a yellow color irrespective of age, adult or infant, while those of premature infants were pigmented without exception, although clinically the infants were not jaundiced. That the pigmentation was not due to postmortem transudation was indicated by the coloration not being present in unjaundiced older patients at a comparable postmortem time. Under spectrophotometric study, it was found that sunlight-protected vitreous humor had an absorption spectrum characteristic of human serum. The spectrum showed a maximum at 460 nm. due to bilirubin, the maxima of oxyhemoglobin at 576 nm. (α band), 540 nm. (β band), 414 nm. (Soret band), and strong protein absorption at 280 nm. Exposure of the vitreous humor to room light and air for several hours resulted in the expected diminution of the 460 nm. band due to photo-decomposition of bilirubin. The features of the absorption spectrum were otherwise modified to show photo-oxidative replacement of oxyhemoglobin with methemoglobin, as seen by the characteristic Soret band at 404 nm. and absorption at 540 nm. (Band III) of the latter protein. The absorption intensity of the methemoglobin bands I, II, and IV were too weak to be documented with certainty. The spectral identification of the vitreous humor pigments as being the serum pigments bilirubin and oxyhemoglobin were confirmed by positive results of the benzidine method for oxyhemoglobin determination and the Jendrassik modification of the Van der Berg diazo reaction for bilirubin. The identity of bilirubin was also indicated histochemically by the Hall staining method.

An attempt was made to quantify the presence of bilirubin in the vitreous humor and serum of
premature infants, and therefore get an estimate for the blood/vitreous human barrier for this compound. The Jendrassik method was used, giving the results in Table I below. Oxyhemoglobin was quantified by the benzidine method. Unfortunately, the low levels of bilirubin in the vitreous humor were difficult to quantitate with the sensitivity of the Jendrassik method, and therefore the figures as given can only be regarded as estimates of the amount of bilirubin present in the ocular humor. Also, the quantity was not calculated from spectral absorption, due to overlap in the absorption of bilirubin and oxyhemoglobin.

**Histochemical staining.** To date, no histochemical staining technique has been successful in displaying bilirubin in neural tissues in states of hyperbilirubinemia. The Hall method, the most sensitive staining method for bilirubin, has also been unsuccessful in detecting this pigment, even in kernicterus, because of the low intracellular levels of bilirubin. The Hall method has never been applied to the retina however, where, as will be described later, it might be expected that bilirubin is present in higher concentrations than in the brain. In the past, only the natural yellow staining of the pigment within brain cells has been noted.

In our work, the H & E staining of the eye sections was uninformative; the yellow color of the bile looked for in the retinal cell cytoplasm was too pale to be detected from the surrounding staining. In the Hall method, bilirubin is stained green. Hall method staining of sections of premature infant eyes showed a highly positive reaction for the vitreous humor which was colored a bright green. Examination of the cytoplasm of the cells of the neural-retina showed a fine green stippling which was uniform for all the cells of the retina under ×400 magnification. Examination of an unstained section showed a fine yellow stippling in the cytoplasm of the retinal cells. This appeared as a black stippling when observed with a Zeiss No. 4720 interference filter, which would be expected for bilirubin. These results, although strongly positive for the presence of bilirubin in the vitreous humor bathing the retina, can only be taken as suggestive that bilirubin is taken up by the cells of the neural-retina.

**Discussion.** The effects of bilirubin on the ocular tissues have never been considered in the past, and no known pathological state has been thus far correlated with hyperbilirubinemia. If bilirubin has an effect on any ocular tissue, one would probably expect such an effect in the developing retina of the premature infant. Bilirubin is a cytotoxic compound to neural cells which acts to uncouple oxidative phosphorylation in the mitochondria. Levels of bilirubin of 20 mg. per cent in the plasma of term infants, and perhaps lower in premature infants, have been correlated with nerve cell damage in the brain.\(^7\) Levels of bilirubin in contact with the retinal cells are expected to be high in premature infants for several reasons. Since the conjugating capacity of his liver is immature, the hyperbilirubinemia is strongly represented by unconjugated bilirubin, which is the form that can easily penetrate the cell membrane. Also, since unconjugated bilirubin is associated with its carrier, albumin, more bilirubin can make contact with the retinal cells than cells of the brain since the blood/vitreous humor barrier is more permeable to plasma proteins than is the blood/CSF barrier.\(^8\) One third of the plasma protein entering the vitreous humor is albumin, and this protein is greatest in concentration in the cortex of the vitreous body.\(^9\) Most importantly, the premature infant's retina is immature, with the process of myelination being incomplete. Silberberg and co-workers\(^10\) have recently demonstrated the tendency of bilirubin to cause cessation of active myelination of cerebellar cells of newborn rats in cell culture. Reasons exist, therefore, for concern over bilirubin toxicity to the retina in the premature infant. We have tried to determine histochemically whether any uptake of bilirubin by the neural retinal cells could be demonstrated, but as mentioned earlier, our preliminary results are at best suggestive that such uptake does take place. The question of neural retinal cell uptake of bilirubin is deserving of further consideration and should be undertaken using more sensitive techniques such as in vivo injection of C\(^{14}\)-bilirubin in newborn animals with autoradiographic analysis, or careful cytological separation with microspectrophotometric analysis of retinal cells utilizing the characteristic bilirubin absorption at 460 nm.

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**Key words:** hyperbilirubinemia, bilirubin, vitreous humor, premature infants, myelination, jaundice.

**Table I**

<table>
<thead>
<tr>
<th></th>
<th>Bilirubin (mg. %)</th>
<th>Oxyhemoglobin (mg. %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Direct</td>
</tr>
<tr>
<td>A Serum</td>
<td>3.4 ± 0.2</td>
<td>0.8 ± 0.14</td>
</tr>
<tr>
<td>Vitreous humor</td>
<td>0.4 ± 0.1</td>
<td>0.2 ± 0.06</td>
</tr>
<tr>
<td>B Serum</td>
<td>27.6 ± 0.6</td>
<td>—</td>
</tr>
<tr>
<td>Vitreous humor</td>
<td>1.2 ± 0.2</td>
<td>0.2 ± 0.06</td>
</tr>
</tbody>
</table>

A. Premature infants (17).
B. Adult (1).
REFERENCES